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# Effects of Electrical Stimulation and Testosterone in Translational Models of Facial Nerve Injury

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LOYOLA UNIVERSITY CHICAGO

EFFECTS OF ELECTRICAL STIMULATION AND TESTOSTERONE IN  
TRANSLATIONAL MODELS OF FACIAL NERVE INJURY

A DISSERTATION SUBMITTED TO  
THE FACULTY OF THE GRADUATE SCHOOL  
IN CANDIDACY FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY

NIJEE SHARMA

CHICAGO, ILLINOIS

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## LIST OF ABBREVIATIONS

A $\beta$	amyloid-beta
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
AR	androgen receptor
BDNF	brain derived neurotrophic factor
CAP23	23 kilodalton cytoskeletal-associated protein
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DC	direct current
DHT	dihydrotestosterone
DI	dorsal intermediate
DL	dorsal lateral
DLN	dorsolateral nucleus
DPO	days post-operative
DM	dorsal medial
ES	electrical stimulation
FGF	fibroblast growth factor
FMN	facial motor nucleus
GAP43	43 kilodalton growth-associated protein
GFAP	glial fibrillary acidic protein
GDNF	glial derived neurotrophic factor
HSP	heat shock protein

HPO	hours post-operative
NGF	nerve growth factor
NT	neurotrophin
PACAP	pituitary adenylate cyclase-activating peptide
PIP2	phosphoinositol 4,5-diphosphate
RT-PCR	reverse transcription-polymerase chain reaction
PNS	peripheral nervous system
SBMA	spinal bulbar muscular atrophy
SD	standard deviation
SDN-POA	sexually dimorphic nucleus of the preoptic Area
SMF	stylomastoid foramen
SNB	spinal nucleus of the bulbocavernosus
TP	testosterone propionate
TRKB	tyrosine receptor-kinase B
TTX	tetrodotoxin
VM	ventral medial
VL	ventral lateral
WPO	weeks post-operative

## **ABSTRACT**

Among the various peripheral nervous system injuries seen clinically, facial nerve lesions are prevalent and have significant functional and emotional impact on patients. As injuries can occur in different segments of the facial nerve and lead to different pathophysiological outcomes, animal models that mimic the common sites of injury need to be developed so that potential therapies can be appropriately investigated. The extratemporal facial nerve axotomy model, in which the nerve is crushed at its exit from the skull, has been well established in the past and used to study the regeneration program of motoneurons. The present study uses this rat injury model to evaluate the therapeutic potential of two treatments, testosterone and nerve electrical stimulation (ES), alone and in combination.

Results demonstrate that ES reduced the delay before sprout formation begins and led to rapid upregulation of regeneration-associated genes, testosterone accelerated the overall regeneration rate and led to delayed but more sustained gene upregulation, and the combinatorial treatment strategy had the most enhanced effects on regeneration events. A more clinically relevant model of facial nerve injury, in which the nerve is crushed during its course in the temporal bone, was also developed and found to lead to substantially prolonged functional recovery times as compared to an extratemporal facial

nerve crush. Finally, the combinatorial treatment of ES plus testosterone accelerated functional recovery following the more proximal, intratemporal crush injury. In conclusion, the present study characterizes two models of facial nerve injury and suggests that a combinatorial treatment strategy of ES plus testosterone holds significant clinical potential.

# **CHAPTER I**

## **INTRODUCTION**

Among the various peripheral nervous system (PNS) injuries seen clinically, facial nerve lesions occur commonly and have significant functional and emotional impact on patients. Due to the nerve's complex anatomical course, injuries can occur in different segments of the nerve, leading to different pathophysiological outcomes. Animal models that mimic the common types of facial nerve injuries seen clinically need to be investigated so that potential therapies can be appropriately developed. The extratemporal facial nerve axotomy model, in which the nerve is crushed at its exit from the skull, has been well established in the past and used to study the regeneration program of motoneurons. The present study uses this model in rats to investigate the therapeutic potential of two treatments, testosterone and electrical stimulation (ES), alone and in combination. Gonadal steroids such as testosterone have been shown to have significant neuroprotective and neurotrophic effects, and ES has also recently emerged as a potential therapy for enhancing nerve regeneration. Our preliminary data shows that following an extratemporal facial nerve crush injury in rats, the use of a combinatorial treatment strategy of ES plus testosterone has additive effects on accelerating functional recovery as compared to the administration of either treatment alone. To delineate the mechanism underlying these effects, this dissertation investigated the effects of ES and testosterone

on the rate of facial nerve regeneration and regulation of regeneration-associated genes following an extratemporal facial nerve crush injury. The second half of this dissertation focuses on development of a more clinically relevant model of facial nerve injury, in which the nerve was crushed during its course in the temporal bone. This intratemporal facial nerve injury model was compared to the pre-existing extratemporal facial nerve injury model. Finally, the neurotherapeutic potential of ES and testosterone was then investigated in the newly developed intratemporal model of facial nerve injury. Corticosteroids such as prednisone are commonly administered in cases of facial nerve injury, although their use remains controversial. Therefore, the effects of prednisone treatment was also compared to ES and testosterone treatments in the intratemporal model of facial nerve injury.

**Aim 1: Determine if daily ES and/or testosterone propionate (TP) affect facial nerve regenerative properties following an extratemporal facial nerve crush in rats.**

*The working hypotheses were that ES and TP independently increase rate of facial nerve regeneration, that ES also shortens the time delay before initial sprout formation begins, and that the combination of ES and TP has an enhanced effect on regenerative properties.* Gonadectomized adult male underwent right facial nerve crush axotomies near the stylomastoid foramen (SMF) and were left untreated or received daily ES and/or TP capsules. The rats were divided into four experimental groups: 1) no treatment 2) ES-only, 3) TP-only, and 4) ES + TP. For each group, radioisotopic labeling of fast axonally transported proteins was used to analyze the outgrowth distance of facial nerves at 4- and

7-days post-axotomy and to calculate the regeneration rates and estimated time delay before sprouting begins.

**Aim 2: Determine if daily ES and TP differentially affect regulation of regeneration-associated genes following an extratemporal facial nerve crush in rats.**

*The working hypothesis was that regeneration-associated genes are significantly upregulated in animals receiving ES or TP and that the upregulation occurs earlier with ES as compared to TP treatment.* Gonadectomized adult male rats underwent right facial nerve crush axotomies near the stylomastoid foramen (SMF) and were divided into four experimental groups: 1) no treatment, 2) ES-only, 3) TP-only, and 4) ES + TP. Punches of facial motor nucleus (FMN) were harvested at various time points following axotomy (6 h, 1 d, 2 d, 7 d, and 21 d), and real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze fold change in mRNA levels. The candidate genes analyzed included  $\alpha_1$ -tubulin,  $\beta_{II}$ -tubulin, 43-kilodalton growth-associated protein (GAP-43), brain-derived neurotrophic factor (BDNF), pituitary adenylate-cyclase activating peptide (PACAP), and neuritin.

**Aim 3: Compare functional recovery and cell survival following an extratemporal facial nerve crush as compared to an intratemporal facial nerve crush in rats.**

*The working hypothesis was that an intratemporal crush of the facial nerve leads to a significantly prolonged recovery time and a greater loss of facial motoneurons as compared to the extratemporal injury.* For the rat intratemporal facial nerve injury model, the facial nerve was crushed during its course through the facial canal in the temporal bone, whereas for the extratemporal injury, the nerve was crushed near its exit

form the SMF. Daily facial functional recovery, weekly motor nerve conduction, and facial motoneuron survival at 4 weeks post-operative were compared between the two injury models.

**Aim 4: Determine the effects of one-time ES, TP, and prednisone on functional recovery following an intratemporal facial nerve crush axotomy.** *The working hypothesis was that the administration of ES and/or TP will shorten recovery time even after a more proximal, intratemporal injury.* Immediately after the intratemporal injury, rats were either left untreated or divided into various treatment groups: one-time ES treatment immediately after injury, TP, or prednisone, a treatment currently used in cases of facial nerve paralysis. The effects of the combination of the various treatments was also be investigated. Daily facial functional recovery and weekly motor nerve conduction were compared among the following experimental groups: 1) control, 2) prednisone-only, 3) ES-only, 4) TP-only, 5) ES + prednisone, 6) TP + prednisone, 7) ES + TP, and 8) ES + TP + prednisone.



## **CHAPTER II**

### **LITERATURE REVIEW**

#### **A. INTRODUCTION**

Peripheral nerve lesions are common types of nervous system injuries, often leading to substandard functional recovery. Although neurons within the peripheral nervous system (PNS) have the ability to regenerate their injured axons, recovery is dependent upon the degree of cell loss, rate of regeneration, and successful reinnervation. The facial nerve is a commonly injured nerve in humans and thus can be used to design treatment strategies that enhance regeneration. Injury to the facial nerve can result from a variety of causes, the most prevalent being Bell's palsy and compression or stretching of the nerve due to tumors or bone fractures. The susceptibility of the nerve to injury arises from its complex anatomical course from the brainstem to its muscles of innervation. As the severity of facial paresis and the recovery outcome is dependent upon the site of facial nerve injury, development of animal models representing these different injury sites will be beneficial not only in characterizing the degree of regeneration but also in testing the effects of potential treatments. Gonadal steroids and electrical stimulation (ES) are two interventions that have the potential to be translated to the clinic.

## B. PERIPHERAL NERVOUS SYSTEM INJURY

Whereas the central nervous system (CNS) usually cannot regenerate after injury, the mature peripheral nervous system (PNS) has a robust ability to regenerate because of activation of its intrinsic growth capacity and a permissive environment (Vargas and Barres, 2007). Despite this regenerative capacity, functional recovery following peripheral nerve injury is often suboptimal. Recovery outcomes vary depending on the proximity of lesion to the cell body, regeneration distance to target, and age of patient (Fu and Gordon, 1995a; Fu and Gordon, 1995b; Birch and Raji, 1991; White and Vaughan, 1991; Vaughan, 1992). Peripheral nerves are anatomically arranged with fatty protection at locations that are vulnerable to mechanical perturbations, but nerves are nonetheless frequently damaged by physical insults, such as disruption of the myelin sheath causing conduction blocks or severe forces that crush, stretch, or sever the nerve.

### 1. ANATOMY OF A PERIPHERAL NERVE

The outer sheath of an entire peripheral nerve consists of condensed layers of connective tissue called the epineurium (Sunderland, 1991; Lee and Wolfe, 2000). Each peripheral nerve trunk is composed of multiple bundles or fascicles of nerve fibers. As the epineurium encircles and runs between fascicles, its main function is to nourish and protect the fascicles. Each nerve fascicle is surrounded by a well-defined sheath known as the perineurium. The perineurial sheath contributes to nerve tensile strength. Within the fascicles, each individual nerve fiber and its investing Schwann cell is surrounded by the endoneurium. The endoneurium functions to protect and nourish the axons.

A complex network of blood vessels serves as the blood supply of a peripheral nerve. There are two major arterial systems, one lying superficially on the nerve and the other lying within the interfascicular epineurium (Sunderland, 1991). The two systems are linked to a minor longitudinal system by anastomoses. The endoneurial capillaries are structurally and functionally similar to the capillaries of the central nervous system in that they act as an extension of the blood-brain barrier. The tight junctions connecting the endothelial cells of these capillaries create a system impermeable to a wide range of macromolecules. Injury, ischemia, or toxins can disrupt this barrier.

## 2. INJURY CLASSIFICATIONS

In 1972, Seddon first classified nerve injuries into three major groups: neurapraxia, axonotmesis, and neurotmesis. Neurapraxia is characterized by local myelin damage that usually occurs as a result of nerve compression. Axonotmesis is defined as a loss of continuity of axons, with variable damage of the endoneurial and perineurial sheaths but an intact epineurium. Lastly, neurotmesis is the loss of the epineurial sheath and the complete physiologic disruption of the entire nerve trunk (Seddon, 1972). After injury, function fails in the following sequence: motor, proprioception, touch, temperature, pain, and sympathetic (Lee and Wolfe, 2000). Recovery usually occurs in the reverse order. Incomplete injuries are more frequent than complete severance. Furthermore, mixed nerve injuries, in which all fibers are affected but to varying degrees, are fairly common.

### 3. CLINICAL PICTURE: FACIAL NERVE INJURIES

Facial nerve injuries are common PNS injuries seen in the clinic that often lead to unsatisfactory or delayed recovery and thus serve as a target for new therapeutic interventions. As the facial nerve controls the muscles of facial expression, victims of facial paralysis can have various functional problems of the eyelid and facial skin and muscles, in addition to severe emotional disturbances from the resulting facial deformity (Rosson and Redett, 2008; Kiese-Himmel et al., 1993). Because the forehead on the paralyzed side of the face has no wrinkles and is unable to raise that eyebrow, the eyebrow droops and pushes the eyelid tissue down over the eye. Although the eyelid itself can open, it has difficulty closing, leading to increased exposure of the eye, dry eye, infections, and in severe cases, corneal ulcers and loss of the eye from perforation (Kerrebijn and Freeman, 1998). In addition, patients present with a flattened nasolabial fold and the corner of the mouth turned down. Other complications can include drooling, speech difficulties, and inability to retain airway patency (Rosson and Redett, 2008). Another common complication in patients with facial nerve injury is synkinesis, or the involuntary movement of part of the face during the voluntary movement of another part of the face.

#### a. Anatomy & Etiology

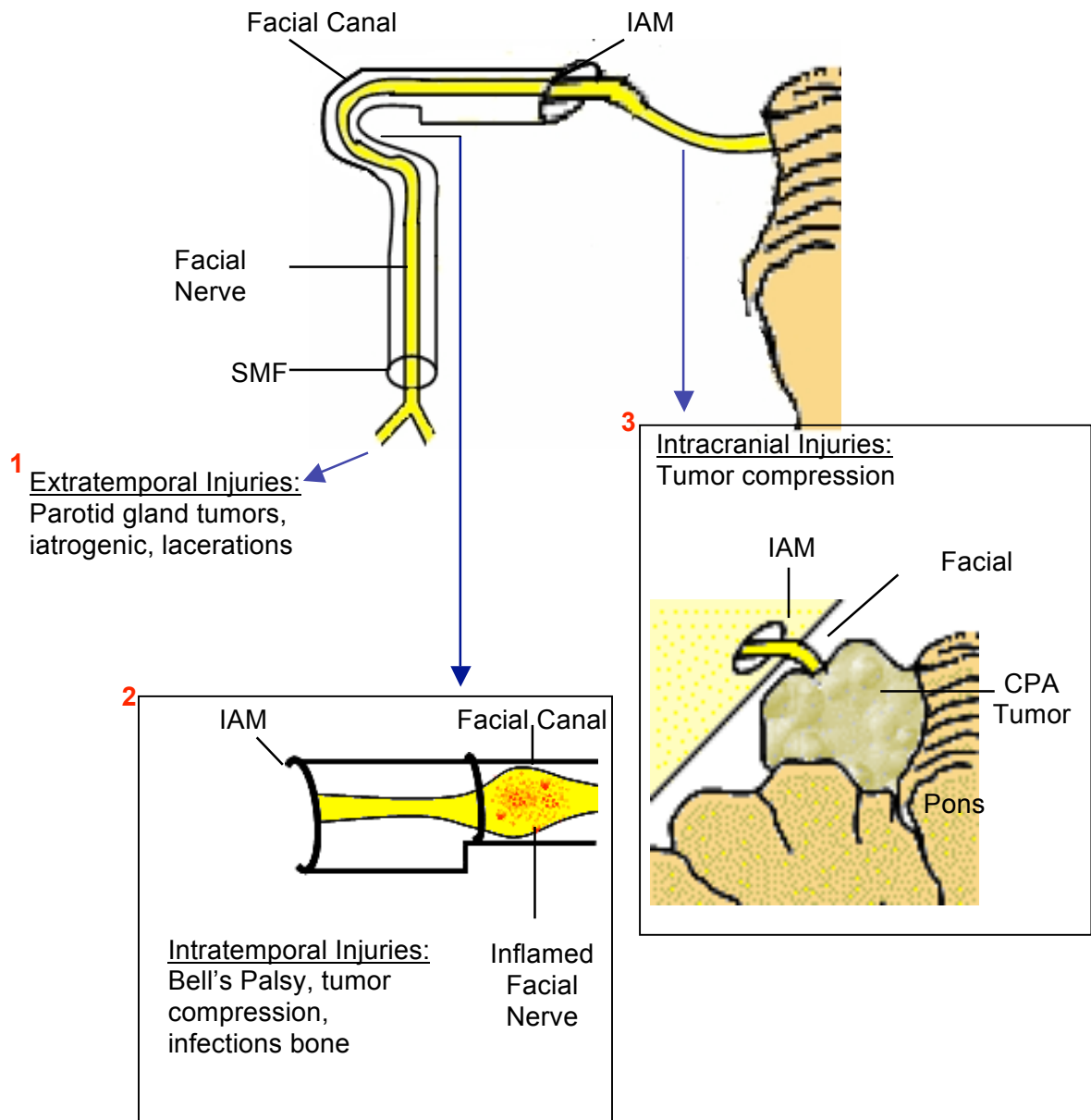
The three main segments of the facial nerve are intracranial, intratemporal, and extratemporal. The intracranial segment of the nerve arises near the pons, travels in the cerebellopontine angle and enters the temporal bone through the internal auditory meatus (Figure 1). In the temporal bone, the nerve travels a complex course confined within a prolonged bony canal that is in some cases not much greater in diameter than the nerve

itself (intratemporal segment). The nerve exits the skull at the stylomastoid foramen (SMF), becoming extratemporal, and is then embedded in the parotid gland in its course. It exits the parotid gland, after branching into five main divisions and many smaller divisions, to finally reach the facial muscles. The course of the facial nerve through the posterior fossa, temporal bone, and parotid gland renders it vulnerable to many neoplastic, traumatic, and infectious events.

Lesions of the facial nerve within the cerebellopontine angle and the internal auditory meatus are usually due to compression by acoustic neuromas, meningiomas, epidermoids (sebaceous cysts), or tumors of the jugular vein (Mavrikakis, 2008; Rosson and Redett, 2008). The nerve may further be damaged during surgical removal of neoplasms. Lesions of the facial nerve within the temporal bone are due to Bell's palsy, skull fractures, spread of inner ear infections, and petrous-temporal cancer. Bell's palsy is the most common, sudden cause of facial nerve paralysis and is believed to be caused by inflammation of the facial nerve during its course in the temporal bone, leading to compression and possible ischemia and demyelination (Tiemstra and Khatkhate, 2007). Although the inflammatory process is not completely understood, reactivation of the herpes simplex virus is thought to be the underlying cause. Extratemporal injuries of the facial nerve may result from traumatic injuries (lacerations and gun shot wounds) and complications arising from parotid gland tumors.

#### b. Evaluation & Management

The gold standard for grading facial nerve function is the House-Brackmann grading scale (House and Brackmann, 1985). The grading system relies on evaluation of



**Figure 1: Anatomy of the facial nerve.** Upon exit from the facial nucleus, the facial nerve runs intracranially, then enters the internal auditory meatus (IAM), turns in the facial canal, and exits the mastoid bone at the stylomastoid foramen (SMF). Different types of injuries can occur at different segments of the nerve: **1**) extratemporal **2**) intratemporal and **3**) intracranial. CPA: cerebellopontine angle.

resting symmetry of the face, degree of voluntary movement on the affected side, and degree of synkinesis, with scores assigned from 1-6 in increasing severity of dysfunction. The degree of nerve damage is often also assessed by nerve conduction studies. A reduction in the compound muscle action potential is suggestive of axonal degeneration, and an increase in latency is indicative of demyelination of the nerve (Kimura, 2006).

Several medical and surgical interventions are currently used for facial nerve injuries. Incomplete paralysis has an excellent prognosis and is managed purely by observation (Danner, 2008). Following complete facial nerve paralysis, the use of corticosteroids is suggested to minimize nerve edema and degradation in nerve function. Whether corticosteroids should be administered, however, is still controversial, as their efficacy has not been clearly demonstrated (Turk-Boru, 2005). Diagnosis of Bell's palsy is usually also followed up with a course of anti-virals (i.e. acyclovir) due to the proposed viral etiology, but studies establishing their beneficial effects in animals and humans are still lacking (Tiemstra and Khatkhate, 2007). Current surgical interventions include decompression, anastomoses, and nerve grafting. If nerve function is significantly compromised at 2 weeks post-injury, decompression of the facial nerve is recommended (Danner, 2008). In this procedure, the intratemporal portion of the facial nerve is decompressed to relieve pressure on the nerve from the surrounding bone. When the facial nerve is transected or sufficiently injured, requiring that the injured portion be removed, an end-to-end anastomoses is performed (Danner, 2008). When additional nerve length is needed, an intervening nerve graft is used. Even though these surgeries re-establish connection between the ends of the facial nerve, regeneration of axons across

the suture site may be hindered. Therefore, the need to develop better treatments prevails as suboptimal functional recovery is often seen.

### C. NEURONAL RESPONSE TO INJURY

Understanding the pattern of events that take place after a peripheral nerve injury can help develop potential treatments that enhance regeneration and recovery following facial nerve injury. Complete repair of damaged axons depends upon a sequence of events: degeneration of nerve fibers distal to the injury site, development of growth cones proximal to the injury site, and regeneration of nerve fibers to reconnect with the original target (Ide, 1996). The rate at which these events occur determines the recovery outcome. A remarkable feature of the mature PNS is its ability to mount a robust regenerative response following injury. Signals of cellular injury and stress are followed by activation of transcription factors, adhesion molecules, growth-associated proteins and structural proteins required for axon elongation (Makwana and Raivich, 2005; Bisby and Tetzlaff, 1992).

#### 1. WALLERIAN DEGENERATION

In 1850, Augustus Waller discovered that following lesion of a nerve, the distal segment undergoes progressive degeneration (Waller, 1850). Calcium influx, after disruption of the axonal integrity, signals resealing of the severed end by a vesicle-mediated process (Krause et al., 1994). Distal to the site of injury, there is beading and swelling of the axonal membrane (George et al., 1995). Following formation of these axolemmal blebs, the axonal cytoskeleton breaks via a process termed granular



disintegration, involving disassembly of microtubules, neurofilaments, and other cytoskeletal components (Vargas and Barres, 2007). Recent studies using transgenic mice expressing the yellow fluorescent protein have shed insight into the spatial progression of granular disintegration. Following nerve transection, disintegration progresses anterogradely, while a crush injury leads to retrograde degradation (Beirowski et al., 2005). After axonal degeneration, blood-tissue permeability increases, the myelin sheath breaks down, and macrophages infiltrate the site of injury (Chen et al., 2007). Macrophages, along with Schwann cells, contribute to the clearance of myelin debris distal to the site of axonal injury. In mammals the process of Wallerian degeneration takes approximately 7-14 days (Choi and Dunn, 2001).

At the cell soma, a critical component of the axon reaction is loss of afferent synaptic boutons from the surface of injured neurons (Graeber et al., 1993; Svensson and Aldskogius, 1993). This phenomenon is known as “synaptic stripping” and is reversible following target reinnervation. Microglia contribute to active stripping of detached presynaptic boutons (Blinzinger and Kreutzberg, 1968). The amount of synaptic stripping correlates with the degree of stress produced by injury, depending on the type and proximity of injury. In neurons disconnected from their target, removal of afferent inputs leads to significant alterations in the electrical properties of neurons and in turn may be related to an initial period of shock (Pinter and Vanden Noven, 1989). Treatments that target synaptic stripping may reduce neural stress and initiate regeneration earlier.

## 2. AXONAL REGENERATION

Whereas the distal axonal stump undergoes degeneration, the proximal stump begins regeneration. Although the signals initiating the regeneration program have not been definitely identified, the disruption of retrograde flow of signals, calcium influx, and exposure of the injured axon end to the degenerating and inflammatory environment play a role in activating regeneration (Chen et al., 2007). Peripheral axon lesions are thought to activate the intrinsic growth capacity by increasing levels of intracellular cyclic adenosine monophosphate (cAMP; Qiu et al., 2002a). Not only are cAMP levels found to be elevated after injury, but injection of dibutyryl-cAMP into the dorsal root ganglia has been found to promote regeneration (Neumann et al., 2002; Qiu et al., 2002b). The mechanism of action for cAMP involves protein kinase A (PKA; Neumann et al., 2002; Qiu et al., 2002). The effects of cAMP and PKA on regeneration are dependent upon transcription, requiring activation of the cAMP response element binding (CREB) protein (Cai et al., 2002; Gao et al., 2004). Activation of a coordinated pattern of gene expression is a crucial factor behind the intrinsic growth capacity of the PNS.

Functionally successful regeneration also depends upon a permissive environment and axon guidance cues that aid axons to regrow toward correct targets. The tip of the regrowing axons, or the growth cone, responds to contact guidance cues, searching for an appropriate matrix and environment to support axonal growth (Chen et al., 2007; Lee and Wolfe, 2000). Schwann cells play an important role in directing regeneration. Additionally, extracellular matrix proteins, such as laminin, mediate interaction between the growth cone and the Schwann cell membrane to promote neurite outgrowth and axonal guidance.

### 3. MOLECULAR GROWTH PROGRAM

Following axonal injury, several changes take place within the neuronal cell body that aid in the mounting of a regenerative response. The cell body swells and undergoes chromatolysis, a process in which the Nissl granules become widely dispersed and the nucleus is displaced peripherally (Choi and Dunn, 2001). These changes together reflect an increase in cellular metabolism, with a shift in priority from synthesis of neurotransmitters to production of structural materials and growth-associated proteins needed for axonal repair and regrowth. Thus, axonal injury shuts down the differentiated functions of an adult neuron and activates a growth program instead (Snider et al., 2002). Over the years, the pattern of gene expression during regeneration has become increasingly well characterized. Strategies designed to boost or accelerate the molecular growth program activated following injury can potentially be used as treatments.

Signals of cellular injury and stress are followed by induction of transcription factors, adhesion molecules, cytoskeletal components, cytokines, and neurotrophic factors (Makwana and Raivich, 2005). One of the earliest changes is the upregulation of polyamine-producing enzymes, linked to an increase in mRNA metabolism and protein synthesis (Tetzlaff et al., 1988a; Gilad et al., 1985; Gilad et al., 1996). Blockade of polyamine synthesis thwarts the normal regenerative process. Cell adhesion molecules are also upregulated following axonal injury. Laminin as well as its receptor,  $\alpha 7$   $\beta 1$  integrin, have been shown to influence the speed of axonal regeneration and reinnervation of peripheral target (Werner et al., 2000). A deletion in the laminin gene results in a significant decrease in the number of axons crossing the injury site to reach the distal portion of the crushed nerve. Another cell adhesion molecule, galectin-1,

stimulates migration of Schwann cells to promote formation of cellular bridges that allow axonal growth into the distal part of the injured nerve (Fukaya et al., 2003; McGraw et al., 2004).

Successful axonal regeneration is accompanied by an increase in cytoskeletal-associated proteins such as actin and microtubule subunits (Makwana and Raivich, 2005). At the growth cone, regulation of actin polymerization and microtubule assembly is crucial for axon elongation. Therefore, axonal injury also leads to increased expression of various functionally diverse molecules that regulate cell surface and cytoskeletal interaction. The 43-kilodalton growth-associated protein (GAP-43) and 23-kilodalton cytoskeletal-associated protein (CAP23) sequester the membrane phospholipid phosphoinositol 4,5-diphosphate (PIP2) and regulate its availability at the raft regions of the cell membrane (Bomze et al., 2001; Laux et al., 2000). Unbound PIP2 promotes actin polymerization. Initially, upregulation of GAP43 and CAP23 may help to sequester PIP2 at the axonal shaft and prevent early axonal branching. Later, phosphorylation of GAP43 and CAP23 may lead to release of PIP2, thus promoting actin polymerization at the edge of the growth cone. Additionally, GAP43 and CAP23 modify recruitment of signaling molecules to rafts, thus altering cell surface interactions. As these molecules also interact with calcium/calmodulin, they may translate calcium fluxes into signals that guide growth cone motility (Dunican and Doherty, 2000; Gerendasy, 1999).

A crucial feature of the transcriptional program activated following injury is the increase in neurotrophic factors. The concept of neurotrophic factors was first put forth by Ramon Y Cajal (Cajal, 1991). Neurotrophic factors promote development and maturation during the embryonic period and maintain neuronal activity during adulthood

and regeneration (Terenghi, 1999). The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and a variety of neurotrophins (NT), NT3 to NT7 (Terenghi, 1999; Gordon, 2009). Axonal injury in the PNS upregulates endogenous neurotrophins in neurons and in denervated Schwann cells. Motor and sensory systems show distinctive patterns of expression of neurotrophic factors. NGF, BDNF, NT-4, and the common neurotrophin receptor, p75, are upregulated in primary sensory neurons following injury (Terenghi, 1999; Funakoshi et al., 1993; Meyer et al., 1992). Upregulation of BDNF, glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and NT-3 is associated with injury to motoneurons (Yan et al., 1992; Sendtner et al., 1990; Zhou and Rush, 1995). Expression of tyrosine receptor kinase B (trkB), the neurotrophin receptor that binds specifically to BDNF and NT-4/5, also increases in motoneurons.

BDNF increases survival of motoneurons following axotomy in neonates and ventral root avulsion in adults (Yan et al., 1992; Novikov et al., 1995; Novikova et al., 1997a; Novikova et al., 1997b). BDNF administration also reverses soma atrophy and prevents reduction of choline acetyl transferase (Kishino et al., 1997). Administration of CNTF promotes muscle fiber reinnervation, preservation of muscle mass, and myelination of regenerating axons (Ulenkate et al., 1994; Newman et al., 1996; Sahenk et al., 1994). NT-3 has been shown to enhance neurite outgrowth in spinal cord explants and to increase the number of motor end-plates *in vitro* (Braun et al., 1996). Like BDNF, GDNF also maintains motoneuron survival, reduces axotomy-induced soma atrophy, and prevents the decline in choline acetyl transferase (Henderson et al., 1994; Matheson et al., 1997). Exogenously administered BDNF and GDNF have been shown to promote axonal

sprouting shortly after axotomy (Boyd and Gordon, 2002). In conditions of prolonged axotomy, BDNF and GDNF also increase the number of neurons regenerating their axons into the distal nerve stump (Boyd and Gordon, 2002; Boyd and Gordon, 2003). In addition to neuronal and non-neuronal cells, neurotrophic factors can also be synthesized in the target muscle tissue.

#### D. NEUROTHERAPEUTIC ROLE OF GONADAL STEROIDS

A variety of hormones, including gonadal steroids, glucocorticoids, and thyroid hormones, have been explored as neurotherapeutic agents for the injured or diseased nervous system. Extensive studies on gonadal steroids have distinguished them as potential therapeutic agents. Estrogen is known not only to affect learning, memory, behavior and cognition, but has also been demonstrated to have neuroprotective and neuroregenerative effects (Green et al., 1997; McEwen and Alves, 1999; Matsumoto and Arai, 1981; Garcia-Segura et al., 2001). Progesterone has been shown to promote recovery from spinal cord injuries and to rescue motoneurons from degeneration (Thomas et al. 1999; Labombarda et al., 2003; Gonzalez et al., 2002). Androgens have been shown to play a neurotherapeutic role in various disease conditions and to enhance peripheral nerve regeneration, as described below.

##### 1. ANDROGENS

Targeting numerous body tissues, androgens have a multitude of functions, including functions in reproduction, development of primary and secondary sexual characteristics, increasing muscle mass, and influencing neuronal functioning. During

nervous system development, the presence or absence of androgens determines the sex-specific morphological and behavioral patterns of the adult (Kaiser and Morley, 1994; Beyer and Hutchison, 1997). Within the brain, testosterone not only exert long-term organizational (permanent) effects during the developmental period, but they also have transient activation (reversible) effects during regenerative events (Arnold and Gorski, 1984).

Gonadal steroids are derived from cholesterol and synthesized primarily in the adrenal cortex and the gonads (Champe and Harvey, 1994). The majority of testosterone production takes place in the Leydig cells of the testes. After secretion from its sites of synthesis, testosterone is delivered via protein-bound transport in the blood stream to target organs. Approximately 60-70% of testosterone is tightly bound to sex hormone binding globulins (SHBG), 30-40% is bound loosely to albumins, and 0.5-2% is free (Iqbal et al., 1983). Sex differences in SHBG influence the circulating levels of testosterone in males and females (Bialek et al., 2004). Also, SHBG levels are known to increase with age, thereby reducing the levels of free testosterone (Leifke et al., 2000). Testosterone, in its free form, can cross the blood brain barrier and have effects on the CNS and the PNS (Champe and Harvey, 1994). As it is lipid soluble, the hormone can easily cross the cell membrane to bind to its intracellular receptor, the androgen receptor (AR). Testosterone can act either directly through an androgen pathway or indirectly through its aromatization to estrogen.

## 2. MECHANISM OF ACTION

Steroid hormones act on the nervous system using two different mechanisms, genomic and non-genomic. The genomic mechanism is slow-acting and involves binding of the hormone to its nuclear receptor. Subsequent binding of the hormone-receptor complex to DNA sites called hormone response elements acts to alter gene expression (Beato, 1989). The non-genomic mechanism is fast-driven and involves interaction of the hormone with membrane or neurotransmitter receptors (McEwen, 1991; Falkenstein et al., 2000). Steroid utilization of one or both of these mechanisms leads to changes in cells of the nervous system and effects on neurotropism and regeneration.

The presence of nuclear ARs in various regions of the brain and spinal cord allows testosterone to exert genomic effects. The distribution of ARs has been identified autoradiographically, biochemically and with *in situ* hybridization and immunocytochemical methods (Morrell and Pfaff, 1978; McEwen et al., 1982; Simerly et al., 1990). ARs are predominantly found in the medial hypothalamic area, ventromedial and dorsomedial nucleus, preoptic area, arcuate nucleus, amygdala, regions of the hippocampus, and lower motoneurons of the brainstem and spinal cord (Ozawa, 2005; Sar and Stumpf, 1977; Yu and McGinnis, 2001). There are sex-related differences in AR distribution, with males having higher concentration of the AR protein than females (Lu et al., 1998). Exposure to gonadal steroids induces rapid ultrastructural changes in neurons, including alterations in the nucleolus and rough endoplasmic reticulum, increases in cell size, increases in nuclear size and changes in nuclear shape from ellipsoid to spherical (Cohen and Pfaff, 1981; Jones et al., 1990). The interval between



rRNA transcription and processing is also shortened in the presence of gonadal steroids (Kinderman and Jones, 1993).

Recent evidence also points towards a non-genomic mechanism of action for gonadal steroids. Extranuclear ARs have been identified in neuronal axons and dendrites as well as glial processes of the cerebral cortex, hippocampus, and amygdala (DonCarlos et al., 2003; Tabori et al., 2005; DonCarlos et al., 2006). The function of extranuclear ARs remains unknown. Wagner et al. (1998) has suggested that the estrogen-receptor complex retains its function as a transcription factor to act on mitochondrial DNA. Also, mitochondrial DNA is known to contain several genes with androgen response elements in the promoter region (Solakidi et al., 2005). Another non-genomic role of androgens may be to activate signal transduction pathways. Androgens have been demonstrated to activate mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 (PI3) kinase pathways in various cell culture systems, including hippocampal neurons, epithelial cells, and skeletal muscle cells (Nguyen et al., 2005; Baron et al., 2004; Estrada et al., 2003). In male rat osteoblasts, testosterone also increases intracellular levels of  $\text{Ca}^{2+}$  within 5 s by increasing  $\text{Ca}^{2+}$  influx and  $\text{Ca}^{2+}$  mobilization from the endoplasmic reticulum (Lieberherr and Grosse, 1999).

In the mouse facial motor nucleus (FMN), AR protein is localized to the cytoplasm but becomes concentrated to the nucleus upon treatment with testosterone (Tetzlaff et al., 2007a). Therefore, androgens may have genomic as well as non-genomic effects within the FMN. Unbound steroid receptors are known to complex with heat shock proteins (hsp; Pratt and Toft, 1997). AR has been shown to associate with hsp70 in prostatic cell lines (Veldscholte et al., 1992). Thus, the administration of androgens may

mediate the cell response following neuronal injury by binding to ARs and releasing hsp70. Previous studies from our laboratory have shown that hsp70 mRNA levels increase by 364% following facial nerve transection in hamsters (Jones et al., 2000). The administration of testosterone, however, prevents this increase, suggesting that it substitutes the need to synthesize hsp70 by making available pre-existing hsp70. This mechanism would allow injured neurons to mount a regenerative response more immediately, without the need to mount a stress response first.

### 3. NEUROTROPHIC EFFECTS

It is now well understood that gonadal steroids have a variety of trophic effects on the nervous system. Studies have demonstrated their effects on cell survival, neurotransmission, growth and development, elaboration of processes, and synaptogenesis (Nordeen et al., 1985; Luine, 1985; Jones et al., 1985; Matsumoto et al., 1988a; Matsumoto et al., 1988b). Androgen-mediated regulation of synaptic connectivity and neurite outgrowth has been well documented in the sexually dimorphic cluster of spinal motoneurons (SNB or spinal nucleus of the bulbocavernosus), which innervate the perineal muscles in the male rodent (Breedlove and Arnold, 1980, Kurz et al. 1986). For example, following castration, the number of synaptic contacts in the SNB decreases, along with a reduction in dendritic length and soma atrophy (Breedlove and Arnold, 1981). These changes are reversed by androgen replacement, suggesting that androgens regulate neuronal function in adulthood (Matsumoto et al., 1988b).

In addition to the SNB, the dorsolateral nucleus (DLN) is another motor neuron population in the rodent spinal cord that is sexually dimorphic. Autoradiographic studies

demonstrated that adult DLN motor neurons concentrate androgens and ARs (Breedlove and Arnold, 1980). In both the SNB and DLN, perinatal exposure to androgens attenuates the cell loss that normally occurs during development in rodent females (Breedlove and Arnold, 1981; Sengelaub and Arnold, 1989). Another well-studied example of androgens effects on cell survival is the sexually dimorphic nucleus of the preoptic area (SDN-POA). Male rodents have larger cells and a greater percentage of cells than females, while neonatal females treated with androgens demonstrate increased volume of SDN-POA (Gorski et al, 1978; Jacobson et al., 1981; Murakami and Arai, 1989). The effects of androgens on survival of non-sexually dimorphic spinal motor neurons have also been demonstrated. Lumbosacral spinal cords of developing embryos cultured in the presence of androgens exhibit an increased number of cells fated to become ventral motor neurons (Hauser and Toran-Allerand, 1989).

#### 4. ROLE IN NEUROBIOLOGICAL DISEASES

Increasing evidence demonstrates that gonadal steroids play a neuroprotective role in diseases of the nervous system. Testosterone levels gradually decrease as a normal consequence of aging in men, and this may make the brain more susceptible to neurodegeneration (Bialek et al., 2004). Glial fibrillary acidic protein (GFAP) has been shown to increase with aging in several regions of the brain, including the hippocampus, striatum, cerebral cortex, and white fiber tract (Nichols et al., 1993). Changes in GFAP expression reflect reactive astrocyte hypertrophy and are often used as a marker for neurodegeneration. Testosterone replacement in 24-month old rats is reported to reverse the age-related increase in GFAP expression (Day et al., 1998). Testosterone

replacement in aging mice also alleviates age-related learning and memory impairment (Flood et al., 1995). Castration has also been shown to reduce cognitive function in male dogs (Hart, 2001).

Testosterone is also proposed to play a neuroprotective role in Alzheimer's disease (AD). Hyperphosphorylation of tau protein, a pathological hallmark of AD, can be reduced by testosterone administration in male rats (Papasozomenos, 1997). Secretion of amyloid- $\beta$  ( $A\beta$ ) peptide, a component of plaques found in AD brain, is also decreased following treatment with testosterone (Gouras et al., 2000). Testosterone has also been shown to protect hippocampal cultures from  $A\beta$ -mediated apoptosis through an estrogen-dependent mechanism involving the aromatization of testosterone to estradiol (Pike, 2001). These and other reports implicate that testosterone may be beneficial for prevention or alleviation of AD. Randomized clinical trials have also examined the effects of testosterone on cognitive function in healthy men aged 50-80 years and shown that testosterone enanthate supplementation improves spatial and verbal memory (Cherrier et al., 2001). An additional study reported that testosterone enanthate also improves working memory in healthy volunteers (Janowsky, 2000). The potential of androgen treatment for mild cognitive impairment and AD is worth exploring.

A neuroprotective role of androgens is also associated with motor neuron diseases. Amyotrophic lateral sclerosis (ALS) is a progressive and fatal motor neuron disease, characterized by progressive weakness and atrophy of skeletal muscles. Deficiencies in androgen receptor are linked to ALS as cranial motoneurons III, IV, and VI, which normally lack AR, are spared from degeneration (Williams and Windebank, 1991). Serum levels of free testosterone are also significantly decreased in ALS patients

(Militello et al., 2002). Spinal bulbar muscular atrophy (SBMA) is another motor neuron disease associated with AR that results in progressive weakness and wasting of the muscles of the limbs and face. An abnormal expansion of the polyglutamine tract in the AR gene leads to a toxic gain-of-function, with aggregates leading to obstacles in cellular processes such as trafficking and connectivity (Poletti, 2004; Brooks et al., 1998). A loss-of-function is also thought to result from the SBMA AR mutation, as the elongated polyglutamine tract leads to reduced transcription of AR (Abdullah et al., 1998; Mhatre et al., 1993). A lack of androgenic control of neurite outgrowth in motoneurons may lead to an inability to maintain or recover normal shape and function of dendrites and axons. In fact, immortalized motoneurons expressing mutant AR display short and dystrophic neurites with abnormal branching patterns (Poletti, 2005). Therefore, a further understanding of androgen-mediated neurotherapeutic effects will be valuable to finding treatment strategies for neurodegenerative diseases.

Several studies have also demonstrated that androgen treatment protects against hypoxic-ischemic brain damage. For example, in a study performed on neonatal rats, a hypoxic-ischemic injury causes obvious signs of injury in cortical and hippocampal neurons, including cell organ decreasing, nuclear swelling, chromatic agglutination, mitochondria decreasing and swelling, and an increase in apoptotic cells (Li et al., 2008; Li et al., 2006). In comparison, treatment with testosterone maintains an integrated nuclear membrane, well-distributed chromatin, and abundant cell organelles, and it reduces apoptosis and enhances regeneration.

## 5. ROLE IN PERIPHERAL NERVE REGENERATION

The therapeutic effects of androgens following peripheral nerve injury have been examined in several model systems. Over a course of numerous studies, our lab investigated the role of gonadal steroids in the neuronal reparative process using a hamster facial nerve axotomy model, in which the facial nerve is crushed or transected at its exit from the SMF. Initial studies were aimed at determining the effects of an ester of testosterone, testosterone propionate (TP), on functional recovery following a facial nerve crush in adult male Syrian hamsters (Kujawa et al., 1989). Varying doses and administration methods of TP were tested: 1) subcutaneous injection of TP in sesame oil vehicle every other day, 2) subcutaneous injection of TP in sesame oil vehicle every day, and 3) subcutaneous Silastic implant of TP. Results demonstrated accelerated functional recovery in each TP-treated group, along with a dose-response relationship. Higher and more frequent doses of TP had better effects on functional recovery, with the continuous dose achieved by Silastic implants having the most enhanced effects. Additionally, gonadectomized male hamsters were not different than gonadally intact animals, indicating that normal endogenous levels of testosterone had little or no effect.

The next study examined the effects of TP on rate of facial nerve regeneration following a facial nerve crush at the nerve's exit from SMF and found that testosterone administration increased the regeneration rate by 26-30% (Kujawa et al., 1991). Furthermore, our lab has found that treatment with testosterone metabolites, dihydrotestosterone (DHT) and estrogen, increases regeneration rates (Tanzer and Jones, 1997). The effects of testosterone on molecular events have also been investigated in the facial nerve axotomy model. Results demonstrate that TP selectively upregulates

regeneration-associated genes such as  $\beta$ <sub>II</sub>-tubulin and GAP-43 and decreases expression of GFAP in the FMN (Jones and Oblinger, 1994; Jones et al., 1997a; Jones et al., 1997b). TP administration also has a pronounced effect on synaptic stripping following facial nerve transection in hamsters (Jones et al., 1997c). TP preserves approximately half of the axosomatic synapses that are otherwise removed in untreated animals, suggesting that TP treatment may reduce the initial shock to injured neurons.

Other peripheral nerve injury models also support a neurotherapeutic role for gonadal steroids in nerve regeneration. Yu and Srinivasan (1981) have shown that TP administration enhances hypoglossal nerve regeneration in rats. TP also increases regeneration in rat sciatic motoneurons and accelerates functional recovery following hind limb paralysis (Kujawa et al., 1993; Brown et al., 1999). Vita et al. (1983) have also reported the accelerative effects of androgens on sciatic nerve regeneration in male rabbits. Apart from enhancing axonal regeneration, gonadal steroids also mitigate cell loss following axotomy. Administration of DHT and estrogen reduces loss of facial motoneurons following a facial nerve transection in neonatal hamsters (Huppenbauer et al., 2005). A study by Perez and Kelley (1996) also found that androgen treatment enhanced survival of laryngeal motoneurons in *Xenopus laevis*. Therefore, androgens serve as a potential treatment strategy for peripheral nerve injuries.

#### E. NEUROTHERAPEUTIC ROLE OF ELECTRICAL STIMULATION

Electrical activity is a normal environmental component within the nervous system and plays an important role from development to adulthood. Application of electrical fields affects morphological and functional properties of neurons such as nerve

branching, rate and orientation of neurite growth, rapid sprouting, and guidance during axon regeneration (McCaig, 1990; Borgens et al., 1981; Patel and Poo, 1982; Manivannan and Terakawa, 1994; Borgens, 1999). Electrical stimulation has been explored as a therapeutic strategy for various neurobiological diseases, including movement disorders, seizures, psychiatric disorders, chronic pain syndromes, and spinal cord and peripheral nerve injuries (Wan and Lin, 2009).

## 1. HISTORY OF ELECTRICAL STIMULATION

Early *in vitro* experiments investigated the effects of ES on morphological properties of neurons. For example, Jaffe and Poo (1979) demonstrated that growing nerve fibers react within minutes of exposure to an applied direct current (DC) field and orient themselves parallel to the gradient. McCaig (1990) reported that applying electric field in *Xenopus laevis* further shaped neurite morphology by increasing the number of filopodia at the growth cone and the number of cytoplasmic spines along a neurite shaft and by enhancing nerve branching. Mannivanan and Terakawa (1994) found that brief pulse stimulation of nerve terminals of bovine chromaffin cells and PC12 cells induced exocytosis and rapid formation of filopodia.

A series of studies also examined axonal regeneration with applied DC fields in giant reticulospinal neurons of the brain and spinal cord of a primitive vertebrate, the lamprey *Petromyzon marinus*. Borgens et al. (1981) applied a weak, steady electric current across completely severed spinal cords of lamprey larvae and found the regeneration rate to be enhanced. The regenerated axons were also able to make functional synaptic connections with the caudal end of the injured spinal cord in the



presence of the applied electric gradient. In another study, Roederer et al. (1983) found that one mechanism by which ES may augment regeneration of the severed lamprey spinal cord is by decreasing the initial “die-back” response, or the retrograde degeneration of the proximal axonal stump. Application of steady electric gradients across injured spinal cords of guinea pigs also induced both anatomical and functional recovery. Wallace et al. (1987) extended these findings by studying the effects of DC fields on axonal regeneration following severe compressive injuries in rats and finding significant acceleration of functional recovery.

ES also influences survival of neuronal populations following axotomy in the CNS. ES of the transected optic nerve for 2 h promoted survival of retinal ganglion cells following transection of the optic nerve in rats (Morimoto et al., 2002). Okazaki et al. (2008) have further examined different ES parameters of optic nerve stimulation. While 10 min of ES was found to be ineffective, 30 min of ES was as effective as 1 h and 2 h of ES in enhancing neuronal survival. The frequency of ES also affected neuroprotection, and a frequency of 20 Hz was found to be optimal, with lower (10Hz) and higher (50Hz) frequencies having no beneficial effect. In summary, numerous experiments over the past two decades have established that ES provides directional cues, influences orientation and regeneration *in vitro* and *in vivo*, promotes survival of CNS neurons, and accelerates functional recovery following spinal cord injury.

## 2. EMERGING ROLE IN PERIPHERAL NERVE REGENERATION

Several studies in the 1980s investigated the effects of ES of injured nerves on axonal regeneration. Nix and Hopf (1983) stimulated the crushed motor nerve

innervating the soleus muscle in rabbits for 24 h daily, over a period of 4 weeks.

Contraction parameters and muscle action potential showed accelerated improvement in stimulated animals. Pockett and Gavin (1985) examined varying periods (5-60 min) of ES of the crushed sciatic nerve in rat. Results indicated that 15-30 min of daily ES accelerated the return of the toe-spread reflex, an index of reinnervation of the flexor muscles. Although these studies were essential in establishing that ES accelerated functional recovery following peripheral nerve injury, they did not distinguish whether the results were due to enhanced regeneration of axons or reinnervation of muscle fibers.

Towards this goal, Al-Majed et al. (2000a) used the rat femoral nerve model of transection and surgical repair to assess the efficacy of ES in accelerating axonal regeneration and promoting reinnervation specificity. A frequency of 20 Hz was used, similar to the normal firing patterns of slow motoneurons. Results demonstrated that 1 h to 2 weeks of ES increased the number of motoneurons that had regenerated not only farther from the site of injury but also into the appropriate branch. Further studies using proximal labeling revealed that 1 h ES of the femoral nerve increased the number of axons that regenerated across the suture gap (Brushart et al., 2002). ES has also been shown to promote regeneration of sensory axons and improve their selective reinnervation following peripheral nerve injury (Brushart et al., 2005).

Recently, studies done in rats were translated to humans in whom the median nerve had been damaged from carpal tunnel syndrome (Gordon et al., 2007). In a randomized clinical trial, the median nerve was stimulated for 1h during carpal tunnel release surgery. ES increased the motor unit number estimates and accelerated functional

recovery in patients. Given these findings, ES can be used as a strategy to improve the outcome of peripheral nerve injury.

### 3. MECHANISM OF ACTION

Investigating the mechanism by which ES helps regeneration can add additional insight on how recovery may be promoted. Al-Majed et al. (2000a) determined that the regeneration-associated effects of ES were mediated by the cell body. In this experiment, transected and repaired femoral nerve in rats was stimulated with or without the application of tetrodotoxin (TTX; blocker of voltage-gated sodium channels), at a dose that is effective in completely blocking action potential transmission to the cell bodies of nerves. TTX completely abolished the ability of ES to enhance axonal regeneration, suggesting that ES acts to enhance the growth program initiated by the cell body.

In a subsequent study, Al-Majed et al. (2000b) demonstrated that ES accelerated the upregulation of BDNF and its trkB receptor in axotomized femoral motoneurons of rats. This was followed by an upregulation of GAP-43 and cytoskeletal proteins (actin and  $\alpha_1$ -tubulin) and a downregulation of neurofilaments (Al-Majed et al., 2004). A decrease in neurofilament to tubulin ratio is associated with enhanced regeneration, as neurofilaments interfere with axonal transport of actin and tubulin (Bisby and Tetzlaff, 1992). English et al. (2006) have further demonstrated that the lack of NT-4/5 in transgenic mice abolishes the enhanced regeneration induced by ES. Therefore, an increase in neurotrophic factor production appears to be an essential downstream effect of ES.

ES is believed to mediate changes in expression of regeneration-associated genes by increasing intracellular levels of cAMP (Gordon, 2009). This early increase in cAMP with ES is most likely preceded by increased calcium entry into the cell body. Interestingly, administration of rolipram, a specific inhibitor of the neuronal enzyme that hydrolyses cAMP (phosphodiesterase IV), mimics the enhanced regenerative effects of ES (Gordon, 2009). Besides stimulating neural cells to synthesize regeneration-associated gene products, ES may work through additional mechanisms. Wan and Lin (2009) recently presented the hypothesis that electrical stimulation of injured nerves may work to accelerate the transport of mRNAs to axons. As axonal injury has been shown to activate local protein synthesis, an enhanced capacity to transport mRNAs and locally translate them may improve the reparative response. Therefore, the authors propose that electrical stimulation may modulate the interaction between RNAs/proteins and carriers or regulate microtubule dynamics.

#### F. COMBINATORIAL TREATMENT STRATEGY

Based on the numerous studies reporting that gonadal steroids positively affect nerve regeneration and the emerging studies demonstrating that ES significantly affects neuronal properties, this dissertation aims to examine the therapeutic effects of combining both treatments in facial nerve injury models. The extratemporal model of facial nerve injury is a well-established model that has been used to study the response of neurons and their microenvironment to injury (Moran and Graeber, 2004). In this axotomy paradigm, the facial nerve is crushed or transected at its exit from the SMF. Clinically, this model is relevant only to facial nerve injuries that occur after the nerve's

exit from the skull. As more proximal injuries are exceedingly prevalent in cases of facial paresis, there is a need to develop animal models representing more proximal injury sites. A rodent intracranial model of facial nerve injury has been described, with the facial nerve being transected ~0.5 mm from its exit from the brainstem (Mattson et al., 1999). However, lesions of the facial nerve during its course in the temporal bone comprise a majority of cases that present in the clinic. Thus development of an animal intratemporal model of facial nerve injury would be beneficial to studying degenerative and regenerative responses and their treatment.

#### A. PRELIMINARY FINDINGS

Our laboratory has recently tested the effectiveness of ES and TP, administered alone and in combination, in accelerating functional recovery in a rat extratemporal facial nerve injury model (Lal et al., 2008; Hetzler et al., 2008). Successful crushing of the nerve resulted in complete loss of the eyeblink reflex, vibrissae orientation, and vibrissae movement. Functional recovery was assessed over time by comparing the return of these parameters on the axotomized side to the unaxotomized side.

We found that the recovery time for the onset of the eyeblink reflex, an indicator of initiation of recovery, was shortened selectively by ES (Figure 2A). In the ES-only group and the ES plus TP group, the average time until the onset of eyeblink was  $3.71 \pm 0.97$  days post-operative (dpo) and  $4.14 \pm 0.55$  dpo, respectively. In comparison, the untreated group and the TP-only group showed significantly delayed recovery at  $9.57 \pm 1.86$  dpo and  $8.38 \pm 0.91$  dpo, respectively. The presence of TP, therefore, did not affect onset of the eyeblink reflex. When analyzing the effects of treatments on complete

functional recovery, we found that the percent change in recovery time was similar with both treatments administered alone. Either with ES-only or TP-only, recovery occurred ~8% faster than in untreated animals (Figure 2B). In the presence of both treatments (ES plus TP), however, animals recovered occurred ~22% faster than untreated animals. This significant improvement with the combined treatment may be a result of each treatment affecting different aspects of cellular events associated with regeneration, therefore leading to an overall additive effect.

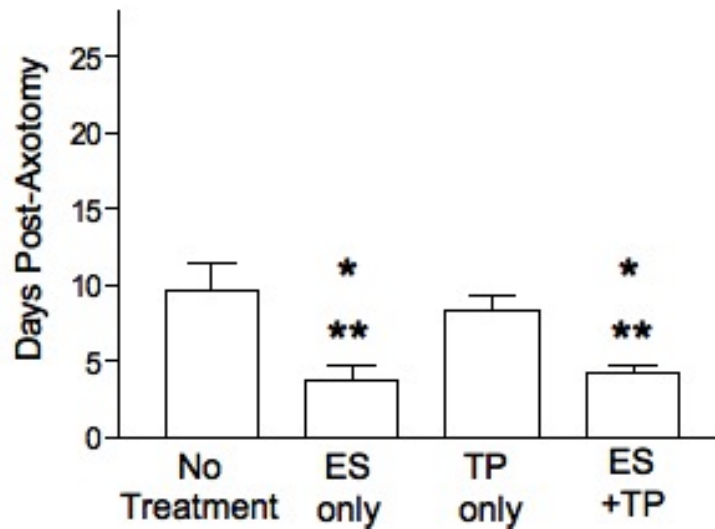
## B. GAPS IN KNOWLEDGE

Further studies need to assess the effects of our combinatorial treatment strategy at the molecular and cellular level to determine if ES and TP work through different mechanisms. A previous study in the hamster extratemporal facial nerve axotomy model has shown that systemic administration of TP accelerates the rate of nerve regeneration but has no effect on the delay before sprouting begins (Kujawa et al., 1991). Whether ES is able to shorten this delay in sprouting remains to be determined. Furthermore, although both ES and testosterone independently have been shown to upregulate regeneration associated genes, it would now be beneficial to compare the differences in specificity and timing of gene expression between the two treatments. If the combinatorial treatment has enhanced effects on axonal regenerative properties and gene expression, then the strategy would demonstrate translational potential for improving recovery following injury of peripheral nerves, such as the facial nerve.

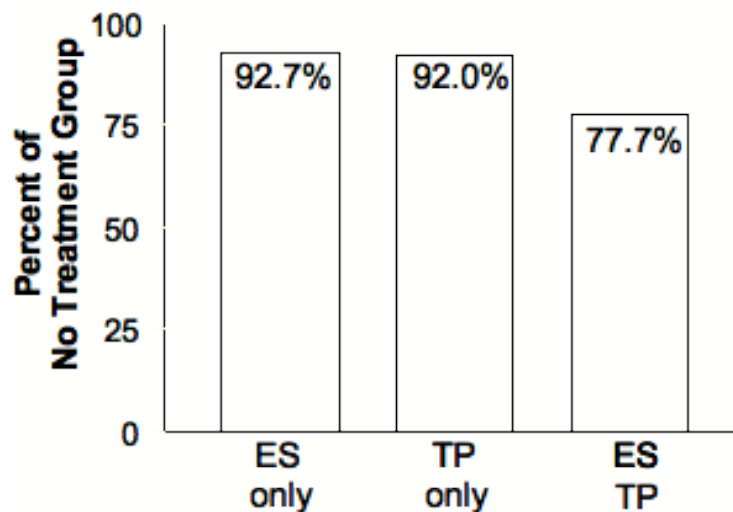
A clinically relevant model of intratemporal facial nerve injury is lacking. Comparing animal models representing two facial nerve injury sites, such as

extratemporal and intratemporal, will allow assessment of how the pathological results and functional outcomes differ between the two. Furthermore, this would provide insight into predicting the course of recovery in patients presenting with the varying facial nerve lesions. **Therefore, the overall goal of this dissertation is to investigate the therapeutic effects of ES and testosterone, alone and in combination, in two translational facial nerve injury models, the well-established extratemporal and the proposed intratemporal.**

### A. Onset of Eyeblink Reflex



### B. Complete Functional Recovery



**Figure 2: Effects of ES and TP on facial functional recovery following an extratemporal facial nerve crush injury in rats. (A)** The effects of ES and TP on the onset of the eyeblink reflex, the earliest functional parameter to return. All data are reported as mean days post-axotomy  $\pm$  the standard error of the mean. (\* =  $p < 0.05$  as compared to no treatment; \*\* =  $p < 0.05$  as compared to TP-only). **(B)** Effects of ES and TP on percent change in complete recovery as compared to the no treatment group. (Adapted from Lal et al. 2008 and Hetzler et al., 2008).



## **CHAPTER III**

### **MATERIALS AND METHODS**

#### **A. ANIMALS**

All animals used in the present study were adult, male Sprague-Dawley rats (2 months old or ~250 g), purchased from Harlan (Indianapolis, IN). Animals were allowed to acclimate to their environment for at least 3 days upon arrival, prior to any manipulation. Rats were housed under a 12 h light/dark cycle in microisolater cages and received a standard rodent diet and water ad libitum. All surgical procedures were completed in accordance with the National Institutes of Health guidelines on the care and use of laboratory animals for research purposes and approved by the institutional animal care and use committee.

Sterile, aseptic techniques were used throughout the study. Any animals displaying  $\geq 15\%$  weight loss were removed from the experiment. Sutures were removed 10-14 days post-operative (dpo). Carbon dioxide asphyxiation was used to sacrifice all animals at the appropriate post-operative survival time.

## B. SURGICAL PROCEDURES

### 1. GONADECTOMY

Gonadectomies were performed 3-5 days prior to facial nerve axotomy. Animals were anesthetized with 3.5% isoflurane, and the surgical area was prepped with ethanol and providone-iodine. A small horizontal incision between the penile and the anal openings was made, and both testicles were pulled through one at a time. The testicular and epididymal arteries were ligated bilaterally with 4-0 silk suture, and testicles were severed distal to the sutures. Wound site was sutured with 4-0 prolene, and animals were allowed to recover under observation.

### 2. EXTRATEMPORAL FACIAL NERVE AXOTOMY

Animals were anesthetized by intraperitoneal injections of Ketamine (100 mg/ml; 0.1 ml/100g body weight) and Xylazine (20 mg/ml; 0.025 ml/100g body weight). The right post-auricular area was shaved and prepped with ethanol and providone-iodine solution. All crush-axotomies were performed on the right facial nerve, with the left facial nerve remaining intact and serving as an internal control in each animal. A small 5 mm incision was made posterior to the right ear cartilage. The underlying muscle layer was carefully dissected away to expose the facial nerve at its exit from the SMF. Under a dissecting microscope, the nerve was gently cleaned to free it from surrounding connective tissue. A fine jeweler's forceps was used to crush the nerve ~1 mm from the SMF. Two 30 sec crushes, from alternating sides, were done to ensure a full crush. (Note: For Aim #3, a single 1 min crush was performed to allow direct comparison to the

intratemporal crush axotomy described below). The injury paradigm severed the axons but left the neural sheath intact to provide a route for regenerating axons. The wound site was sutured with 4-0 prolene and coated with Triple Antibiotic Ointment (Henry Schein). Successful crush was verified by complete loss of the eyeblink reflex and loss of vibrissae orientation and movement in animals upon recovery from anesthesia.

### 3. INTRATEMPORAL FACIAL NERVE AXOTOMY

Animals were anesthetized by intraperitoneal injections of Ketamine-Xylazine, as described above. During surgery, animals were kept on a heating pad to avoid hypothermia. The right post-auricular area was shaved and prepped, and a curvilinear incision was made ~5 mm behind the ear. Under a dissecting microscope, the extratemporal portion of the nerve was exposed at its exit from the SMF, anterior to the sternocleidomastoid muscle. The sternocleidomastoid tendon was divided to provide better exposure of the nerve. The dorsal and caudal aspects of the ear canal were incised so that the incision was immediately lateral to the tympanic membrane. Next, the ridge of bone superior to the temporal bone and the tympanic membrane was identified. The temporalis muscle inserting on this ridge anteriorly and the rhomboid capitis muscle inserting posteriorly were sharply incised with a #15 scalpel blade. The muscles were gently dissected off the ridge, down to the level of the temporal bone, to expose ~1.5 cm of the bone superior to the tympanic membrane.

Next, during drilling of the temporal bone, the facial nerve was constantly kept in view to avoid inadvertent injury. A #3 French suction-irrigator was used to bathe the operative field in room temperature 0.9% saline to prevent thermal injury to the temporal

bone and the facial nerve from the drilling procedure. Using a dental drill and a 3 mm cutting burr, the superior tympanic bulla was drilled in a saucerized manner. The lateral bony aspect of the epitympanic region was also removed to expose the ossicles. The malleus, incus, and stapes were removed with jeweler's forceps to allow access to the region of the temporal bone medial to the ossicles, where the intratemporal portion of the facial nerve lies.

A 2 mm diamond burr was then used to further remove bone from the superior tympanic rim and to thin the antero-superior aspect of the SMF. A 1 mm diamond burr was used as the drill came closer to the underlying facial nerve. Starting at the posterior aspect of the SMF and continuing antero-medially, the bone covering the facial nerve was progressively removed. Vibrissae twitching on the ipsilateral side was observed as the drill came nearer to the nerve. After the bone covering the facial nerve had been sufficiently thinned, a Rosen needle was used to remove any remaining bone from the surface of the nerve. Jeweler's forceps were used to crush the nerve firmly for 1 min. Due to limited access to the nerve, the nerve could only be crushed in one orientation (from top).

Any bleeding during the surgical procedure was controlled with epinephrine-soaked pellets. Gelfoam was lightly packed into the middle ear cavity prior to closure. The external ear canal incision was closed internally using 4-0 silk suture to prevent contamination of the middle ear from the external ear. The muscle and skin were closed in separate layers. Upon recovery from anesthesia, successful crush in all animals was verified by examining loss of the eyeblink reflex and vibrissae orientation and movement.

#### 4. STEREOTAXIC PROCEDURE

Stereotaxic injection of isotopes was performed for the facial nerve regenerative properties study. A 1:1 mixture of tritiated leucine and lysine was prepared as follows. Each labeled amino acid, purchased from GE Healthcare, arrived at a concentration of 10 mCi, in 5ml of ethanol. Next, 500  $\mu$ l of each isotope was combined together in 10 Eppendorf tubes for a final volume of 1000  $\mu$ l in all tubes. A Speedvac was then used to dehydrate the samples. The pelleted isotope in the first Eppendorf tube was redissolved in 100  $\mu$ l of physiological saline. The entire solution was then transferred to the next Eppendorf tube containing a pelleted isotope. This step was repeated for each tube until the final tube contained 1000  $\mu$ l of the leucine-lysine mixture, concentrated to 100  $\mu$ Ci/ $\mu$ l. This tube of isotope mixture was covered with parafilm and store at -20 °C until needed for stereotaxic injection.

Eighteen hours prior to the time of sacrifice, rats were anesthetized with Ketamine-Xylazine, as described in the previous section. The dorsal surface of the cranium was shaved and prepped, and animals were placed in a stereotaxic apparatus. The mouthpiece was adjusted to ensure that the head was flat. A midline incision was made over the scalp, and the soft tissue overlying the skull was gently scraped off to expose bregma. A 26-gauge stainless steel guide cannula was lowered to the surface of the skull, and the tip was placed exactly over bregma. The coordinates (anterior/posterior, medial/lateral, and dorsal/ventral) of the cannula at this position were noted. From here, the stereotaxic apparatus was adjusted to the coordinates of the right facial nucleus in rats (AP: -11.2; ML: -2.2; Paxinos and Watson, 2007). A mark was made on the surface of the skull to denote the position of the guide cannula. The cannula

was then raised, and a 1 mm hole was made in the skull using a dental drill. The guide cannula was lowered into the brainstem, slightly above the location of the FMN (DV = -7.2). A 33-gauge injection cannula, having a 3 mm overhang as compared to the guide cannula, was then placed into the guide cannula and lowered into the brain stem to reach the appropriate FMN coordinate (DV = -10.2). A small digital pump that depressed the plunger of a 10  $\mu$ l Hamilton syringe, connected to cannula tubing (PE 20 intramedic polyethylene tubing), was used to deliver tritiated leucine/lysine. A total of 1  $\mu$ l (100  $\mu$ Ci) of the isotope was injected into the right facial nucleus, at a rate of 0.16  $\mu$ l/min. After injection, pressure within the brainstem was allowed to equilibrate for 5 min before removing the cannula. The hole in the skull was covered with Gelfoam, and the wound was closed with 4-0 prolene suture. Animals were sacrificed 18 h after stereotaxic injection of radioisotope, and axonal regeneration was measured as described in section F.

## C. ELECTRICAL STIMULATION

### 1. DESIGN OF ELECTRODE APPARATUS

A custom electrode apparatus was constructed in our laboratory for implantation in rats. Two Teflon-coated wires (Cooner Wire, Chatsworth, CA) were bared of insulation for 2-3 mm and soldered to two “male” connector pins (Wire Pro) in a connector strip (Allied Electronics). The connector pins soldered to the wires were covered with insulation tubing (Allied Electronics) and epoxy and allowed to dry overnight. One of the wires and its respective connector pin was marked with red insulation tape to designate it as the positive end of the electrode while the other wire and

its connector pin were left unmarked to designate that as the negative end. The connector assembly was then cemented into a syringe base using dental acrylic. Two holes had been drilled on both sides of the syringe base to allow suturing of the electrode apparatus in animals. All electrode apparatuses were tested with a voltmeter to ensure that they were functional prior to use in animals.

## 2. ELECTRICAL STIMULATION IN ANIMALS

Electrodes were implanted in animals at the time of axotomy. A midline incision was made over shaved and prepped skin on the back of animals. The syringe base of the electrode apparatus was sutured using 4-0 silk onto the paraspinal back muscles of rats. The wires were run through subcutaneously, and the positive wire was sutured proximal to the crush site adjacent to the facial nerve, while the negative wire was sutured ~3-5 mm away onto surrounding connective tissue. The connector pins of the electrode were connected to the leads of an isolated pulse stimulator (W-P Instruments, Inc.), and current was directed from the negative to the positive lead. Voltage was increased slowly and set at a threshold at which animals displayed a right ear flutter. The supramaximal, monophasic pulses were delivered at a frequency of 20 Hz. During the stimulation period, animals were allowed to roam freely in a chamber. Unstimulated animals were connected to the stimulator, placed in the chamber, but the voltage was kept at 0 V.

For the regenerative properties study in aim 1, ES was begun 1 dpo and administered daily for a 30 min period until the time of sacrifice. For the gene expression study in aim 2, ES was begun immediately after axotomy and administered daily for a 30 min period until the time of sacrifice. For the intratemporal facial nerve

axotomy model in aim 4, ES was administered at only one time, for a 30 min period, beginning immediately after axotomy. In this paradigm, the electrode apparatus was not implanted in animals, but rather the wires were sutured temporarily in place for the one-time treatment.

## D. STEROID ADMINISTRATION

### 1. TESTOSTERONE PROPIONATE

TP was administered using subcutaneous capsule implants. Capsules were made of Silastic tubing (0.062 in. ID x 0.095 in. OD) and were 18 mm in total length. Of this length, 10 mm contained 100% crystalline TP (Sigma) while 4 mm wooden plugs sealed both ends of the capsule. Prior to use in animals, capsules were equilibrated overnight in physiological saline. In each animal receiving TP treatment, 2 capsules were implanted at the time of axotomy. A subcutaneous pocket for the capsules was created by making an incision on the mid-dorsal surface of animals or using the same incision site used for electrode implantation. Capsules were left in animals until the time of sacrifice. This dose of TP has previously been shown to establish supraphysiological levels of systemic testosterone (Hetzler et al., 2008; Tanzer and Jones, 2004; Kujawa et al., 1989).

### 2. PREDNISONE

A tapering dose of prednisone (Sigma) was administered using oral gavage. Either prednisone dissolved in physiological saline or saline alone was delivered into the esophagus of animals using a syringe connected to 2" curved, 18-gauge gavage needle



(Braintree Scientific). The tip of the gavage needle was lubricated with Puralube prior to insertion into animals. Beginning immediately post-axotomy, a 2.5 mg/kg dose of prednisone was administered as follows: twice daily on 0-2 dpo, once daily on 3-5 dpo, and once every other day on 6-10 dpo. In animals not receiving prednisone, similar volume of saline was injected at the same dosing intervals.

## E. ANIMAL TESTING

### 1. FUNCTIONAL RECOVERY TESTING

In rodents, a complete crush or transection of the facial nerve results in ipsilateral loss of the eyeblink reflex, backwards vibrissae orientation, loss of vibrissae movement, and drooping of the mouth, and return of facial nerve function can be easily observed. Animals were allowed to roam freely in an empty cage, placed on a black background, to observe return of vibrissae orientation and movement. To elicit the eyeblink reflex, a puff of air was blown into the animal's eye and the extent of eyelid closure was observed. Since symmetry of the mouth in rats was not obvious from observation alone and could not be easily scored, improvement in mouth droop was not used as an analysis parameter for facial nerve function.

For all functional recovery studies described herein, function on the crushed, right side was always compared to the intact function on the left side using a 3-point recovery scale. A score of '1' was used to indicate complete lack of function, '2' to indicate the onset of any return in function, and '3' to indicate complete, symmetrical function as compared to the left side. Recovery time for each functional parameter (eyeblink reflex,

vibrissae orientation, vibrissae movement) was defined as the number of dpo until the animals received a score of '3'. For eyeblink function, the number of dpo until the onset of the reflex (score of '2') was also analyzed. Behavioral observations were conducted daily in all animals until a score of '3' was attained for all functional parameters. Complete functional recovery was defined as the number of dpo when all of the parameters had returned to normal.

## 2. NERVE CONDUCTION TESTING

Motor nerve conduction studies were performed weekly, beginning on the day of axotomy and continuing until the day of sacrifice. Animals were anesthetized with intraperitoneal injections of Ketamine-Xylazine, as described in the previous section. A total of 5 electrodes (Grass Technologies, Astro-Med, Inc.) were used for testing. Two stimulating electrodes were placed below the infraorbital ridge to stimulate the facial nerve, while two recording electrodes were placed on the vibrissae pad to record the response. As the vibrissae pad in rats contains organized rows of indentations, the placement of electrodes could be easily replicated for each recording. A ground electrode was placed inferior to the external ear. Electrodes were connected to an electromyographic machine (Advanced Medical) that stimulated and displayed the response. The size of the response (amplitude) in mV and the time in ms (latency) for the electrical impulse to travel from the stimulation to the recording site were the two parameters used to analyze facial nerve conduction.

The optimal current for stimulation of the facial nerve was first determined in test animals. Current was increased to obtain maximal response of the uninjured facial nerve,

thereby ensuring that the current would be high enough to capture any response upon stimulation of the injured facial nerve. By this method, the optimal current intensity was determined to be 15 mA. The facial nerve was stimulated at a frequency of 0.5 Hz, and an average of 25 responses was recorded for each trial. Trials were repeated at least once to verify consistency of the recorded response. Recordings were conducted both on the right and the left sides, and percent change in amplitude and latency for the right facial nerve, relative to the left, was analyzed.

## F. MEASUREMENT OF AXONAL REGENERATION

### 1. FACIAL NERVE DISSECTION & QUANTIFICATION OF RADIOACTIVITY

Eighteen hours following stereotaxic injection of radioisotope, animals were sacrificed at 4- or 7-dpo to analyze axonal regenerative properties. The right facial nerve was carefully dissected out from the SMF to the snout using iridectomy scissors. Nerves were placed on an index card, rapidly frozen with powdered dry ice, and then cut into 1 mm segments using a gel slicer (Mickel Engineering Co., Ltd.). Each nerve segment was then placed into an individual scintillation vial and solubilized overnight in 100  $\mu$ l of Solvabe (Sigma). Next day, 100  $\mu$ l of 10% acetic acid and 5 ml of scintillation cocktail was added to each vial. Radioactivity was assessed on a scintillation counter that was set to count for 3 min on a tritium channel.

## 2. ANALYSIS OF AXONAL REGENERATION

To determine axonal regeneration rate, the radioactivity of each nerve segment was first plotted as a function of distance. The leading edge of the regenerating facial motor axons was defined as the farthest point two standard deviations (SD) above the mean tissue background levels of radioactivity (Oblinger and Lasek, 1984; Forman and Berenberg, 1978). Tissue background was calculated from the most distal nerve segments that were too far from the crush injury site to contain any regenerating axons at the post-axotomy times examined. Outgrowth distance calculated in this manner was then plotted as a function of post-operative time. Two post-operative times, 4 and 7 d, were used. Regeneration rates were calculated from the slope of the line, and extrapolation of the line to the x-intercept provided an estimate for the initial delay in axonal sprouting (Oblinger and Lasek, 1984).

### G. REAL TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Real time RT-PCR was used to assess fold changes in gene expression. Animals were sacrificed at post-operative survival times 0 h, 6 h, 1 d, 2 d, 7 d, and 21 d. Brains were removed and immediately placed, ventral side up, on a rat brain matrix (Redding) on ice to provide support during sectioning. One razor blade was placed at the level of the pontine band, while another was placed 1 mm caudal to it. This coronal section was placed on a Petri-dish on ice, and 1 mm concentric punches of the right and left FMN were taken. Rostral to the site of section, the internal genu of the facial nerve was identified to verify the precise location of the FMN and symmetry between the control

and axotomized sides. Tissue was then homogenized with Lysing Matrix D (MP Biochemicals) and 80  $\mu$ l of Solution D (Sigma).

RNA was extracted using the phenol/chloroform extraction method, precipitated with 100% ethanol, and quantified on a Nano-Drop Spectrophotometer. Samples containing 275 ng of total RNA were treated with DNase I amplification grade (Invitrogen) to remove any genomic DNA. Next, 100 ng of DNA-free RNA was used for the reverse transcription reaction (Superscript II, Invitrogen) to yield 100  $\mu$ l of cDNA samples. Amplification reactions were performed in triplicates using 96-well optical plates and iCycler (Bio-Rad). The total volume of PCR mix per well was 25  $\mu$ l [12.5  $\mu$ l SYBR Green PCR Mix (Applied Biosystems), 10.5  $\mu$ l H<sub>2</sub>O, 1  $\mu$ l gene-specific primer pair (10  $\mu$ M each primer), and 1  $\mu$ l cDNA].

The following rat-specific primers were purchased from SuperArray Biosciences, Inc. and used to amplify cDNA of candidate genes:  $\alpha$ 1 tubulin (Tuba1),  $\beta$ II Tubulin (Tubb2), GAP43, BDNF, PACAP (Adcyap1), and neuritin (Nrn1). Cycling parameters were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles comprising 15 sec at 95°C and 60 sec at 60°C. After PCR amplification, the samples were heated from 60°C to 95°C to perform melt curve analysis and verify the specificity of the amplified products. Fold changes in mRNA levels on the axotomized side as compared to the control side were calculated using the  $2^{(-\Delta\Delta Ct)}$ -1 method, and fold changes were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The cycle threshold (Ct) represents the PCR cycle number at which the increase in fluorescence (and therefore cDNA) is exponential. In the equation  $2^{(-\Delta\Delta Ct)}$ ,  $\Delta\Delta Ct = \Delta Ct_{(axotomy)} - \Delta Ct_{(control)}$ . Here,  $\Delta Ct_{(axotomy)}$  is the Ct value for axotomized sample

normalized to the endogenous housekeeping gene and  $\Delta C_{t(\text{control})}$  is the  $C_t$  value for the control sample normalized to the endogenous housekeeping gene.

#### H. TISSUE SECTIONING AND CELL COUNTS

Animals were sacrificed at the appropriate post-operative survival time, and brains were removed, flash frozen in *n*-butyl bromide/2-methylbutane, and stored at -80°C. Brains were allowed to equilibrate at -20°C at least 24 h prior to cryosectioning. At -20°C, serial 40  $\mu\text{m}$  cryostat sections were collected through the entire caudal to rostral end of the FMN onto Superfrost Plus slides (Fisher Scientific), and slides were stored at -20°C. Prior to histological processing, slides were allowed to equilibrate to room temperature for 20 min. Sections were first fixed in 4% paraformaldehyde, in 0.01 M phosphate buffered saline, for 15 min. Sections were then stained with thionin for 5-8 min and dehydrated in graded ethanol concentrations (50, 75, 95, and 100%) for 30 sec each. Slides were allowed to clear in Hemo-De (Fisher-Scientific) overnight and coverslipped using Permount (Fisher-Scientific). Coverslipped slides were left lying flat for several days and allowed to dry at room temperature. Slides were organized in correct order for each animal and given to a second investigator for counting under blind conditions.

Neuronal survival in the FMN was counted using the Neurolucida software. The abducens nuclei and the internal genu of the facial nerve were used to match the location of the left (control) and right (axotomized) FMN. Only surviving facial motoneurons, with a clearly visible nucleus, were counted. Cell profile counts were taken for every 40

µm section of each rat. The number of facial motoneurons on control and axotomized side were totaled and compared to obtain percent survival.

## I. STATISTICAL ANALYSES

All statistical analyses were performed using the GraphPad Prism software. To determine significant differences between two groups, a two-tailed Student's *t*-test with unequal variances and  $p < 0.05$  was performed. To determine significant differences between three or more groups, a one-way analysis of variance (ANOVA), with a Newman-Keuls' multiple comparison post-hoc test at  $p < 0.05$ , was conducted. A two-way ANOVA was done when two independent variables, such as time and treatment, were present. This statistical test was again followed by a Newman-Keul's multiple comparisons post-hoc test to determine specific differences among groups.

**CHAPTER IV**

**EFFECTS OF ELECTRICAL STIMULATION AND TESTOSTERONE ON  
REGENERATIVE PROPERTIES FOLLOWING  
EXTRATEMPORAL FACIAL NERVE INJURY**

**A. ABSTRACT**

As functional recovery following peripheral nerve injury is dependent upon successful repair and regeneration, treatments that enhance different regeneration events may be advantageous. The neurotherapeutic effects of nerve electrical stimulation and gonadal steroids have independently been demonstrated. The purpose of the first aim of this dissertation was to investigate the therapeutic potential of combining ES with gonadal steroids on peripheral nerve regeneration. Following a facial nerve crush axotomy near the nerve's exit from the SMF in gonadectomized adult male rats, TP was systemically administered with/without daily ES of the proximal nerve stump. Facial nerve outgrowth was assessed at 4 and 7 dpo using radiolabeling of fast axonally transported proteins. Administration of ES alone reduced the estimated delay in sprout formation but failed to accelerate the overall regeneration rate. Conversely, TP treatment alone accelerated the regeneration rate by ~10% but had no effect on the sprouting delay. Combining TP with ES, however, maintained the enhanced rate and reduced the sprouting delay. The findings suggest that as ES and TP differentially enhance



properties, the combination of both may boost regenerative properties more than the use of either treatment alone.

## B. INTRODUCTION

Peripheral nerve lesions are common types of nervous system injuries, often leading to substandard functional recovery. Although the peripheral nervous system has a robust ability to regenerate, recovery from injured nerves is dependent upon cell survival, repair, and mounting of a successful regenerative response. Despite available techniques to surgically repair transected peripheral nerves, complete functional recovery is often hindered by slow rates of regeneration over long distances and/or inadequate target reinnervation (Valero-Cabre et al., 2004). Strategies to increase the rate of axonal regeneration and recovery are thus valuable therapeutic approaches.

It has been well established that gonadal steroids have significant trophic effects on the nervous system, including changes in cell survival, neurotransmitter metabolism, and neuronal regeneration (Schumacher et al., 1996; De Nicola, 1993; Jones, 1994; Jones, 1993a; Tetzlaff et al., 2006; Bialek, et al., 2004). Accordingly, gonadal steroids have been explored as a therapeutic strategy for improving the outcome of peripheral nerve injury. Over a course of several studies, our lab has used the well-established facial nerve axotomy model, in which the nerve is crushed extratemporally at its exit from the SMF, to demonstrate that exogenously applied testosterone accelerates functional recovery and regeneration rates in hamsters (Kujawa et al., 1989; Kujawa et al., 1991; Jones, 1993b; Tanzer and Jones, 1997). Following an extratemporal facial nerve

transection in neonatal hamsters, we have also found that gonadal steroids (androgens and estrogen) can rescue facial motoneurons from degeneration (Huppenbauer et al., 2005). Studies in other injury models further support the neurotherapeutic role of gonadal steroids. Estrogen and selective estrogen receptor modulators have been reported to stimulate sciatic nerve regeneration in mouse models (Islamov, et al., 2002; McMurray et al., 2003). Testosterone has been shown to accelerate functional recovery from hind limb paralysis following a sciatic nerve crush in rats and also to increase axonal regeneration rates following axotomy of the rat hypoglossal nerve (Brown et al., 1999; Yu, 1982; Yu and Yu, 1983).

Gonadal steroids primarily exert their effects on target tissue using a receptor-mediated mechanism. Although binding of androgens to AR can have some non-genomic effects, the primary mechanism of action for androgens involves binding of the hormone-receptor complex to DNA sites to alter gene expression. Gonadal steroid receptors are found in many populations of neurons within the brain and spinal cord, including most cranial and spinal motor neurons (Yu and McGinnis, 2001; Tetzlaff et al., 2007a). Administration of testosterone at the time of nerve injury has been shown to upregulate expression of regeneration-associated genes such as  $\beta$ II tubulin and GAP-43 (Jones et al., 1997a; Jones et al., 1999). Since receptor-mediated mechanisms require multiple steps for signal transduction, the therapeutic effects of gonadal steroids may be delayed in comparison to therapies that can exert more immediate effects.

ES is one potential intervention that is increasingly being studied in different nerve injury paradigms. Application of electrical fields has long been known to affect

morphological and functional properties of neurons, such as nerve branching, rate and orientation of neurite growth, rapid sprouting, and guidance during axon regeneration (Borgens et al., 1981; Patel and Poo, 1982; Manivannan and Terakawa, 1994; Borgens, 1999). More recently, brief ES of the rat femoral nerve proximal to the injury site was shown to enhance axonal regeneration and specificity of motor re-innervation pathways (Al-Majed et al., 2000). ES also improves reinnervation and modulates spinal plastic changes following sciatic nerve injury in rats (Vivo et al., 2008). Direct ES of the transected rat optic nerve improves survival of retinal ganglion cells, demonstrating that such an intervention can also rescue neurons from degeneration (Morimoto et al., 2002). ES appears to exert these effects by influencing the neuronal soma response, in part by increasing expression of neurotrophic factors and their receptors and elevating levels of intracellular messengers such as cAMP soon after injury (Al-Majed et al., 2000; Al-Majed et al., 2004; Udina et al., 2008).

In the first aim of this dissertation, the effects of a combinatorial treatment strategy, administering both gonadal steroids and ES, on nerve regenerative properties were investigated in the extratemporal facial nerve axotomy model. The rationale for this strategy was that, if the two treatments work through different mechanisms or during different phases of regeneration, then using them in combination may enhance recovery to a greater extent than using either treatment alone. Our previous studies have tested the effects of ES and gonadal steroids, alone and in combination, on functional recovery following a facial nerve crush injury in rats. Results show that while either treatment alone accelerates recovery by ~8%, the combination treatment of electrical stimulation

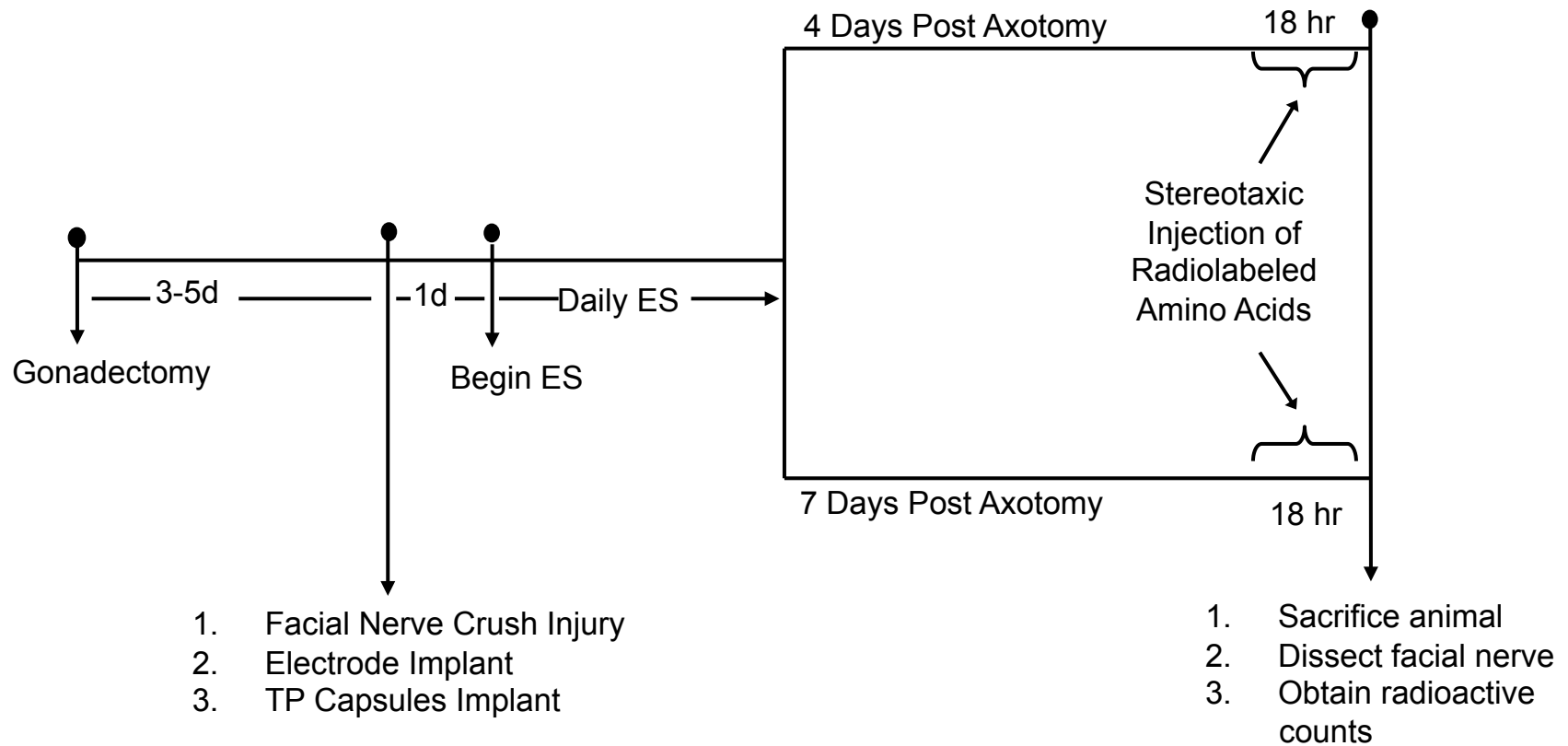
plus TP shortens recovery time by ~22% compared to untreated animals (Lal et al., 2008; Hetzler et al., 2008). In the present study, to further assess the therapeutic potential of ES and TP, we used radiolabeling of fast axonally transported proteins to analyze how the two treatments affect axonal regeneration rates and initial delay in sprout formation following an extratemporal facial nerve crush injury in gonadectomized adult male rats.

## C. MATERIALS AND METHODS

### 1. ANIMALS & NERVE INJURY PARADIGM

Adult male Sprague-Dawley rats, weighing 250g, were purchased from Harlan (Indianapolis, IN) and used for all experiments, as described in Chapter III. Three to five days prior to nerve injury, rats were anesthetized with isoflurane and castrated. For facial nerve axotomy, rats were anesthetized by intra-peritoneal injections of Ketamine (100mg/ml; 0.1ml/100g body weight) and Xylazine (20mg/ml; 0.025ml/100g body weight). The right facial nerve was crushed near its exit from the SMF (refer to Chapter III for details). Two successive 30-second crushes, on alternating sides, were done with fine jeweler's forceps to ensure a full crush.

The experimental design for this study is presented in Figure 3. Animals were divided into 4 experimental groups: [1] no treatment, animals receiving axotomy but no treatment, [2] ES only, animals receiving axotomy and ES treatment, [3] TP only, animals receiving axotomy and TP treatment, and [4] ES + TP, animals receiving axotomy and the combination of ES and TP treatments. For each treatment group, rats



**Figure 3: Experimental Design for Aim 1.** This timeline will be followed for all four experimental groups: no treatment, ES only, TP only, and ES plus TP.

were further divided into two sets of postoperative survival times, 4 and 7 days.

Ultimately, each treatment group had an *n* of 6 animals per time point, for a total of 48 animals in the study.

### 3. ELECTRICAL STIMULATION

A custom electrode apparatus constructed in our laboratory was implanted in all rats (refer to Chapter III). Two Teflon-coated wires, bared of insulation for 2-3mm, were soldered to two “male” connector pins in a connector strip. The connector assembly was cemented into a syringe base using dental acrylic. At the time of axotomy, the base of the syringe was sutured onto the paraspinal back muscles of rats. Wires were run through subcutaneously and sutured ~2 mm proximal to the injury site (cathode) and ~3-5mm away from it (anode). The connector pins of rats were attached to leads of an isolated pulse stimulator, and rats were stimulated at a voltage at which they displayed a right ear flutter. Starting at 1-day post-axotomy, rats were either stimulated with supramaximal pulses delivered at a frequency of 20 Hz or sham stimulated for 30 min daily until sacrifice.

### 4. HORMONE ADMINISTRATION

Immediately after injury, rats receiving testosterone treatment were subcutaneously implanted with two Silastic capsules (0.062 in. id x 0.095 in. od; 10-mm length), equilibrated in physiological saline and containing 100% crystalline TP (refer to Chapter III for details). The dosage given has previously been shown to establish

supraphysiological levels of systemic TP (Kujawa et al., 1989; Tanzer and Jones, 2004; Hetzler et al., 2008).

## 5. STEREOTAXIC SURGICAL PROCEDURE

Eighteen hours prior to sacrifice at 4 and 7 days post-axotomy, anesthetized animals were injected with 1 µl of a 1:1 mixture of  $^3\text{H}$ -leucine and lysine (concentrated to 100 µCi/µl) into the right facial nucleus, as previously described in Chapter III. Animals were placed in a stereotaxic apparatus, a 1mm hole was drilled in the skull, and a stainless steel cannula was placed into the brainstem at the appropriate stereotaxic coordinates for the right facial nucleus (ML = -2.2, AP = -11.2, and DV = -10.2), based on the atlas of Paxinos and Watson (2007). The tritiated leucine/lysine mixture was delivered through the cannula at a rate of 0.16 µl/min. After delivery, pressure within the brainstem was allowed to equilibrate for 5 min before the cannula was removed. The skull hole was filled with Gelfoam and the incision site was sutured.

Eighteen hours following injection of isotope, animals were sacrificed by CO<sub>2</sub> asphyxiation. The right facial nerve was dissected from the SMF to the snout and rapidly frozen. Nerves were cut into 1mm segments using a gel slicer. Each nerve segment was placed into an individual vial and solubilized overnight in 100 µl of Solvable. Next day, 100 µl of 10% acetic acid and 5ml of scintillation fluid was added to each vial, and radioactivity was assessed on a scintillation counter (refer to Chapter III for details).

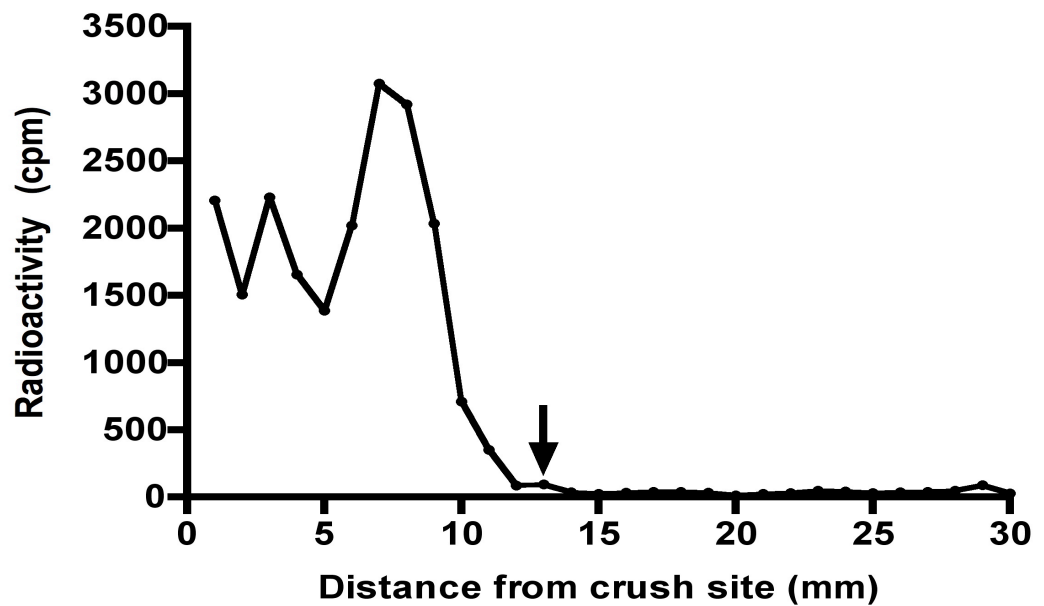
## 6. MEASUREMENTS OF AXONAL REGNERATION & STATISTICAL ANALYSIS

Axonal regeneration was measured as previously described in Chapter III. To determine axonal regeneration rate, the radioactivity of each nerve segment was first plotted as a function of distance. The leading edge of the regenerating facial motor axons was defined as the farthest point  $\geq 2$  SD above the mean tissue background levels of radioactivity (Oblinger and Lasek, 1984; Forman and Berenberg, 1978). Regeneration rates (slopes) were then calculated by plotting outgrowth distance as a function of days post-axotomy. Extrapolation of the line to the x-intercept provided an estimate of the initial delay in axonal sprouting (Oblinger and Lasek, 1984). A two-way ANOVA (factors = treatment and post-operative time) and a *F* test at  $p < 0.05$  was used to assess significant differences between lines (Forman et al., 1980). Significance among outgrowth distances was determined using Newman-Keuls' multiple comparison post-hoc test. For ease of visual display, each treatment group (ES only, TP only, or ES + TP) is graphed separately to compare it to the no treatment group.

### D. RESULTS

The radioisotopic labeling method is a well-established and sensitive assay for measuring axonal regeneration. Radiolabeled amino acids are incorporated into proteins that are fast axonally transported to the growing tips of the regenerating facial motor axons, and the extent of radioactivity along the nerve correlates to the outgrowth distance. Figure 4 shows an example of distribution of transported radioactivity at 4 days



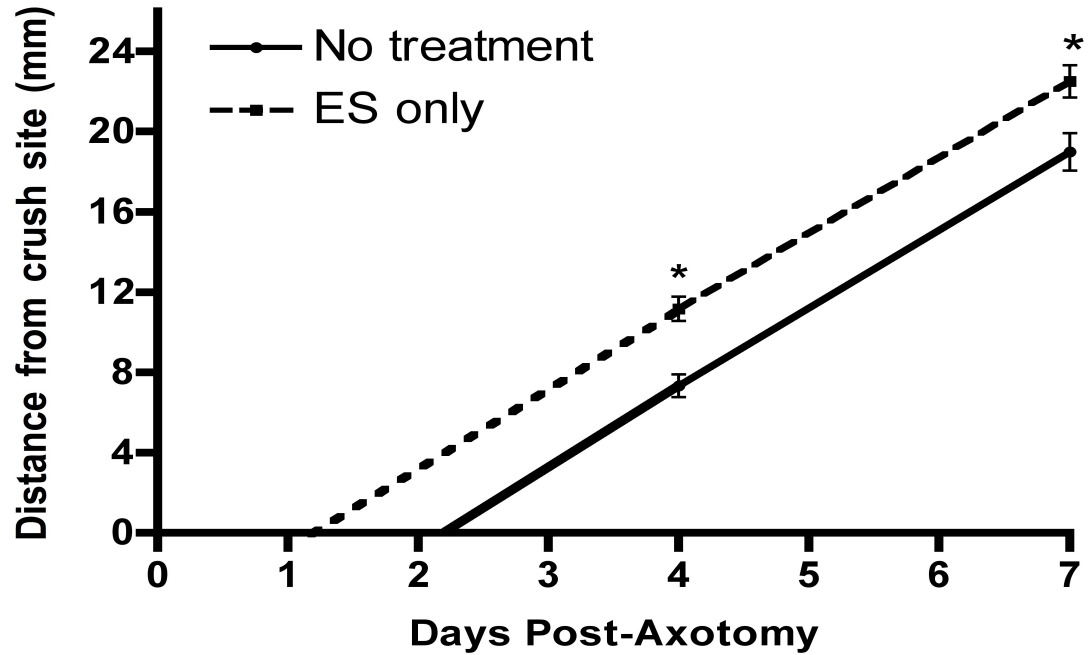


**Figure 4. Distribution of fast axonally transported radioactivity in regenerating facial motor axons at 4 days post-axotomy.** Location of crush site is 0 mm. In this sample graph, the leading edge of the regenerating axon has reached an outgrowth distance of 13 mm. Outgrowth distance is defined as the point that is 2 SD or more above mean tissue background levels calculated from the most distal segments of the facial nerve.

post-axotomy. High levels of radioactivity are present in proximal portions of the regenerating facial nerve, and they fall to a constant low level of background activity distally along the nerve. The leading edge of the regenerating axons is defined as that distance at which the radioactivity is  $\geq 2$  SD above mean tissue background levels (Oblinger and Lasek, 1984), calculated from the most distal segments of the dissected facial nerve that does not contain regenerating fibers. Previous studies using this radioisotopic labeling method to measure regeneration have demonstrated that the rate of axonal elongation is linear (Oblinger and Lasek, 1984; Forman and Berenberg, 1978; Bisby, 1979; Bisby, 1978). Studies in our lab have previously established that regeneration of facial motor axons in rodents is linear as well (Kujawa et al., 1991). Therefore, to conserve the use of animals, outgrowth distances at only two post-operative times were collected to calculate the regeneration rates in the present study.

## 1. EFFECTS OF ES ON FACIAL NERVE REGENERATIVE PROPERTIES

The effects of daily ES of the proximal nerve stump on facial nerve outgrowth of gonadectomized male rats are presented in Figure 5 and summarized in Table 1. Following an extratemporal facial nerve crush injury, the outgrowth distance of the regenerating facial motor axons in untreated animals was  $7.3 \pm 1.0$  mm at 4 dpo and  $19.0 \pm 1.6$  mm at 7 dpo. In comparison, ES significantly increased the outgrowth distance to  $11.2 \pm 1.0$  mm and  $22.5 \pm 1.4$  mm, at both the early and the late time points, respectively ( $p < 0.05$ ; Fig. 5). Based on the outgrowth distances, the untreated animals displayed a regeneration rate of 3.9 mm/d, and extrapolation to the x-intercept indicated an estimated



**Figure 5. Effects of ES on axonal regeneration following an extratemporal facial nerve crush injury in rats.** The mean outgrowth distances at 4 and 7 dpo are plotted as a function of days post-axotomy. The slopes of the lines represent the rate of regeneration while extrapolation of the data points to the x-intercept gives an estimate for the initial delay in sprout formation. Untreated animals and animals receiving only daily ES are shown. Asterisks represent significant differences between the outgrowth distances of groups. Vertical lines represent standard error of the mean.

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**Table 1. Summary of effects of ES and TP on facial nerve regenerative properties**

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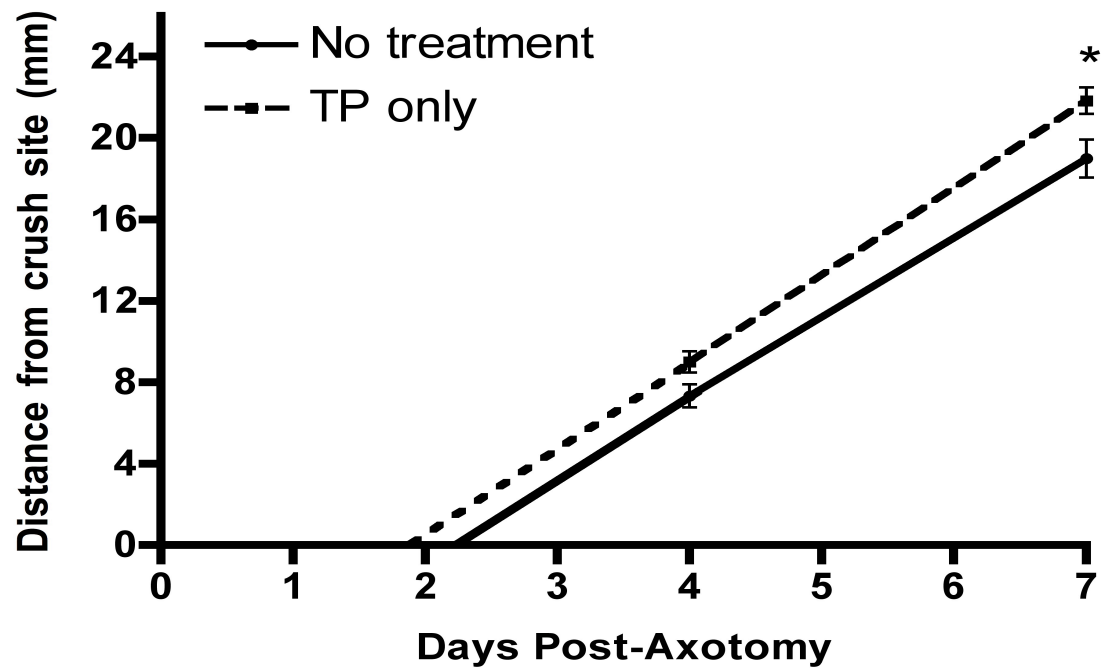
	No treatment	ES only	TP only	ES plus TP
<b>Rate of Regeneration (mm/d)</b>	3.9 <i>n</i> = 12	3.8 <i>n</i> = 12	4.3 <i>n</i> = 12	4.4 <i>n</i> = 11
<b>Delay of Sprout Formation (d)</b>	2.1	1.0	1.9	1.3

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initial delay in sprouting of 2.1 d (Table 1). Daily ES of the facial nerve did not alter the regeneration rate (3.8 mm/d) but did reduce the delay of sprout formation to 1.0 d. Since electrical stimulation did not affect the regeneration rate, the significant increase in outgrowth distances at 4 and 7 dpo was attributed to the reduced delay in sprout formation.

## 2. EFFECTS OF TP ON FACIAL NERVE REGENERATIVE PROPERTIES

Figure 6 compares the effects of TP administration alone to no treatment. The outgrowth distance of the regenerating facial nerve fibers was significantly higher in the presence of TP was  $9.0 \pm 0.9$  mm at 4 dpo and  $21.8 \pm 1.8$  mm at 7 dpo. While the outgrowth distance in TP-treated animals was significantly greater than that of untreated animals at 7 dpo ( $19.0 \pm 1.6$  mm;  $p < 0.05$ ), it was not significantly greater than untreated animals at 4 dpo ( $9.0 \pm 0.9$  mm). Additionally, the outgrowth distance at 4-dpo was also significantly lower in the TP-treated group compared to the group receiving ES only ( $p < 0.05$ ). In contrast to ES, systemic exposure to TP accelerated the regeneration of facial motor axons to 4.3 mm/d (Table 1). This represents a 10% increase in the regeneration rate as compared to untreated animals. TP did not, however, affect the initial delay in sprout formation, which was 1.9 d in TP-treated animals and 2.1 d in untreated animals (Table 1). As the delay in sprout formation was not affected, the increase in outgrowth distance with TP administration may be attributed to a significant acceleration of regeneration rate.



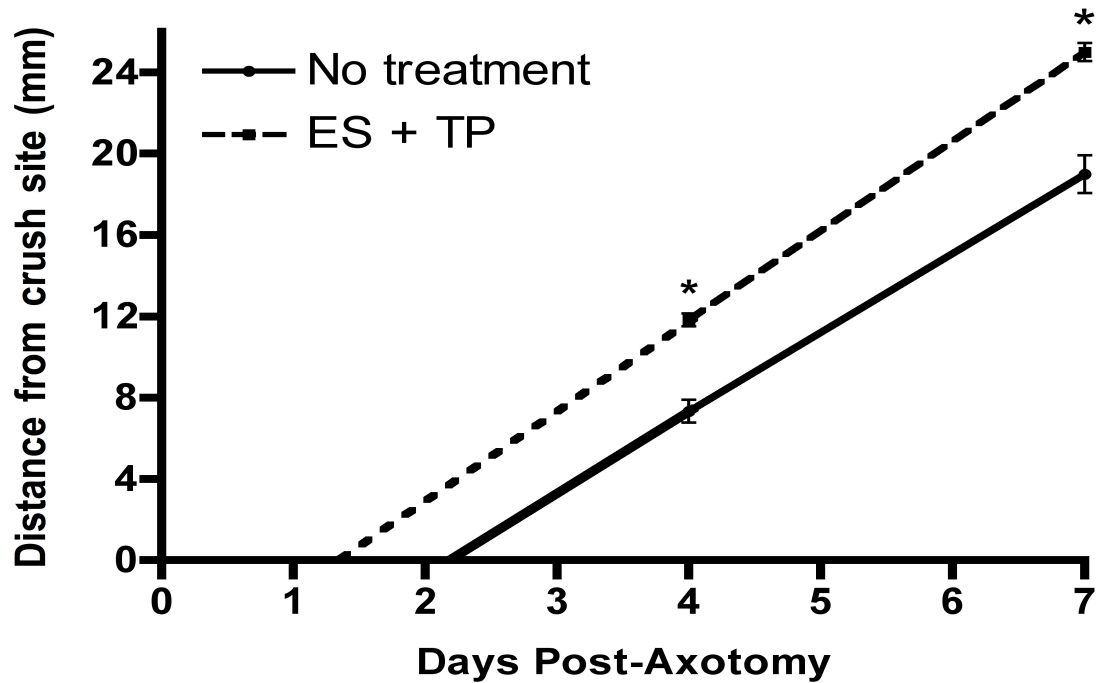
**Figure 6. Effects of TP on axonal regeneration following an extratemporal facial nerve crush injury in rats.** The mean outgrowth distances at 4 and 7 dpo are plotted as a function of days post-axotomy. The slopes of the lines represent the rate of regeneration while extrapolation of the data points to the x-intercept gives an estimate for the initial delay in sprout formation. Untreated animals and animals receiving only systemic testosterone are shown. *Asterisks* represent significant differences between the outgrowth distances of groups. Vertical lines represent standard error of the mean.

### 3. EFFECTS OF ES PLUS TP ON FACIAL NERVE REGENERATIVE PROPERTIES

Since electrical stimulation affected the delay in sprout formation and TP affected the regeneration rate, the combination of both treatments would be expected to have additive effects on axonal regenerative properties. Figure 7 demonstrates that the facial nerve outgrowth distance for the animals receiving the combined treatment was significantly increased to  $11.8 \pm 0.7$  mm at 4-dpo, relative to the outgrowth of  $7.3 \pm 1.0$  mm in untreated animals ( $p < 0.05$ ). At 7-dpo, the facial motor axons of animals receiving the combined treatment had regenerated to  $25.0 \pm 0.7$  mm, farther than not only untreated animals ( $19.0 \pm 1.6$  mm) but also animals receiving only TP ( $21.8 \pm 1.8$  mm) or only ES ( $22.5 \pm 1.4$ ;  $p < 0.05$ ). The animals receiving the combined treatment ES plus TP also demonstrated an increased regeneration rate of 4.4 mm/d and a reduced delay in sprout formation of 1.3 d, relative to a rate of 3.9 mm/d and a delay of 2.1 d in untreated animals (Table 1). Therefore, both the regeneration rate and initial delay before sprout formation begins were beneficially affected with the combined treatment, whereas individual treatment with ES or TP affected only one of the properties.

### E. DISCUSSION

Our previous study demonstrated that administration of TP or daily ES alone improved functional recovery by ~8%, whereas the combination of both accelerated recovery by ~22% (Lal et al., 2008; Hetzler et al., 2008). This study further delineates the mechanism underlying the additive effects of gonadal steroids and ES. While ES of



**Figure 7. Effects of ES plus TP on axonal regeneration following an extratemporal facial nerve crush injury in rats.** The mean outgrowth distances at 4 and 7 dpo are plotted as a function of days post-axotomy. The slopes of the lines represent the rate of regeneration while extrapolation of the data points to the x-intercept gives an estimate for the initial delay in sprout formation. Untreated animals and animals receiving only daily ES and systemic testosterone are shown. *Asterisks* represent significant differences between the outgrowth distances of groups. Vertical lines represent standard error of the mean.



the proximal nerve stump reduced the delay in sprout formation, systemic TP treatment augmented the regeneration rate. Thus, each single treatment had differential effects, but with the combined treatment, both of the regenerative properties were enhanced. Therefore, the therapeutic strategy of using both ES and TP is worthy of clinical consideration in cases of peripheral nerve injury.

Assessment of functional recovery following an extratemporal facial nerve crush injury demonstrated that the onset of the eyeblink reflex occurred significantly earlier in animals receiving ES, suggesting that ES may help initiate early events in axonal regeneration (Lal et al., 2008). Our present results further support this, as ES reduced the initial delay before sprout formation began, thus increasing facial nerve outgrowth distances. However, as ES did not alter the regeneration rate, it may work to enhance functional recovery by promoting early sprout formation rather than changing the rate at which the axon elongates. Application of electric field to nerve terminals has been shown to induce rapid filopodial sprouting in primary neuron cell cultures in the presence of extracellular  $\text{Ca}^{2+}$  (Manivannan and Terakawa, 1994). It has also been reported that, following sciatic nerve crush injury in rats, administration of ES accelerates the appearance of the toe-spreading reflex, a sensitive indicator of the onset of motor recovery (Pockett and Gavin, 1985). Therefore, ES may enhance early regeneration events to initiate sprout formation.

In the literature, there is considerable variability in the initial delay before sprout formation occurs, ranging from 0.4 to 5.2 d, during which a growth program has to be initiated for axonal outgrowth to begin (Oblinger and Lasek, 1984; Forman and

Berenberg, 1978). In the present study, ES of the proximal nerve stump was begun at 1 dpo, and the initial delay in sprouting was reduced from 2.1 d to 1 d post-injury in animals treated with ES. Therefore, ES may have immediate effects on regenerative properties, and if the treatment is initiated on the day of injury, the effects may be even more pronounced. ES is thought to mediate its positive effects on regeneration by enhancing the cell body response to injury. Expression of the neurotrophin BDNF, its high affinity receptor trkB, GAP-43, and T $\alpha$  1-tubulin significantly increase soon after femoral nerve injury in the presence of low-frequency ES (Al-Majed et al., 2000b; Al-Majed et al., 2004). Rather than altering the rate of axonal transport or regeneration rate, as little as 1 h of ES has been shown to accelerate axonal growth across the injury site, thereby reducing the time needed for motor neurons to reinnervate muscles following rat femoral nerve transection and surgical repair (Brushart et al., 2002). Unstimulated motor nerves, on the other hand, show a prolonged delay for axons to cross the injury site. Similar effects of ES in the CNS have also been demonstrated. ES of the sciatic nerve peripherally promotes onset of regeneration of centrally injured dorsal root ganglion axons (Udina et al., 2008). Investigators report that while ES increased axonal outgrowth into the lesion site, it failed to increase axonal elongation rate. Therefore, the results from our present study further support the idea that ES accelerates the onset of regeneration but does not affect the overall rate of axonal regeneration.

To study the effects of gonadal steroids on facial nerve regeneration, we used a standard neuroendocrine paradigm, consisting of removal of the endogenous source of testosterone by gonadectomy and replacement with supraphysiological levels of TP. Our

previous studies using the hamster extratemporal facial nerve axotomy model have shown that axonal outgrowth rates and functional recovery are similar in intact and gonadectomized animals (Kujawa et al., 1989; Kujawa et al., 1991). Rather, it is the administration of exogenous supraphysiological levels of androgens and estrogen that is capable of accelerating axonal regeneration rate. We used gonadectomized instead of intact animals in this study to minimize variability and establish similar baseline levels. Systemic administration of TP increased the regeneration rate by ~10%. Interestingly, TP accelerated functional recovery by a similar magnitude (~8%) as compared to untreated animals (Hetzler et al., 2008). The present results concur with those of previous studies in different models showing that systemic exposure of TP accelerates axonal regeneration rate but fails to reduce the initial delay before sprout formation begins (Kujawa et al., 1991).

The effects of testosterone on axonal outgrowth rates parallel those of conditioning lesion studies, where a first (priming) lesion of a peripheral nerve precedes a second (testing) lesion. Both the conditioning paradigm and gonadal steroids may act by priming the cell body metabolically to enhance the neuronal response to injury and increasing the synthetic capabilities of the regenerating neuron (Tetzlaff et al., 2006; McQuarrie and Grafstein, 1981). It has been shown that the administration of flutamide, an AR blocker, abolishes the effects of testosterone on hamster facial nerve regeneration, indicating that TP works in an AR-dependent manner (Kujawa et al., 1995). Administration of testosterone at the time of facial nerve axotomy in hamsters upregulates the expression of regeneration-associated genes  $\beta_{II}$  tubulin, GAP-43, and

neuritin, associated with neurite outgrowth (Jones et al., 1997; Jones et al., 1999; Fargo et al., 2008a). In addition to using a genomic mechanism to enhance regeneration, androgen treatment may mediate the motoneuron stress response following injury. Heat shock proteins, principal players of the stress response, normally complex with unbound AR in the cytoplasm (Tetzlaff et al., 2007b; Jones et al., 2000). Our lab has previously shown that TP exposure allows HSPs to liberate from ARs, thus reducing the need for the cell to produce more HSPs after injury and instead to direct its metabolic energy towards synthesis of growth-associated proteins.

It must be noted that the regeneration rates presented in this study are for the fastest growing facial motor axons and thus do not represent slower regenerating fibers. Also, the delay times reported are estimations only, as values were obtained by extrapolation of the line to the x-intercept. Quantitative analysis of the regenerating nerve at the ultrastructural level is required to verify the effects of ES and gonadal steroids on the initial delay before sprout formation begins. Our future studies will examine if a short-term ES treatment is just as beneficial as daily stimulation and thus better able to translate clinically. Also, since TP can be converted to estradiol via aromatization and to dihydrotestosterone (DHT) via  $5\alpha$ -reduction, its effects on accelerating the regeneration rate may be attributed to androgenic and/or estrogenic-mediated mechanisms. DHT, in contrast to TP, is a nonaromatizable form of androgen that cannot be converted to estrogen. Therefore, in a subsequent study, our lab investigated the effects of DHT and estradiol, alone and in combination ES, on functional recovery and regenerative properties following an extratemporal facial nerve crush in rats

(Foecking et al., 2009; Sharma et al., 2009). Our findings indicated that neither DHT nor estradiol in combination with ES is as effective as TP in enhancing functional recovery or accelerating regeneration. Therefore, a synergism between androgenic and estrogenic actions may be required to obtain maximal effects in augmenting regeneration. Future studies will examine the effects of the combination therapy of ES and the various gonadal steroids in female rodent models.

Combining ES and gonadal steroid administration had an enhanced effect on regeneration as compared to the use of either treatment alone. The finding that the regeneration rate was similar in TP-treated and combination-treated animals suggests that perhaps ES does not further enhance the molecular mechanism by which TP mediates this effect. Alternatively, the regeneration rate for the rat facial nerve system may already be at its preset maximal level with the administration of TP alone and therefore the use of the combined treatment cannot further increase the rate. Independently, each treatment differentially affected regeneration as ES enhanced immediate events such as sprouting and outgrowth distance at the early time point of 4-dpo whereas TP enhanced the overall regeneration rate. Therefore, the combinatorial treatment strategy of gonadal steroids and ES promises to be a beneficial therapeutic intervention following peripheral nerve injury.

**CHAPTER V**

**EFFECTS OF ELECTRICAL STIMULATION AND TESTOSTERONE ON  
EXPRESSION OF REGENERATION-ASSOCIATED GENES FOLLOWING  
EXTRATEMPORAL FACIAL NERVE INJURY**

**A. ABSTRACT**

As functional recovery following peripheral nerve injury is often suboptimal, treatments that enhance the intrinsic ability to neurons to regenerate may be advantageous. Using the injury paradigm of extratemporal facial nerve crush, our previous study investigated the effects of a combinatorial treatment strategy, consisting of ES of the proximal nerve stump and systemic TP administration in Aim 1. Results indicated that the two treatments differentially enhance facial nerve regenerative properties, whereby ES reduced the delay before sprout formation, TP accelerated the overall regeneration rate, and the combinatorial treatment had additive effects. To delineate the molecular mechanisms underlying such treatments, the second aim of this dissertation investigated the effects of ES and TP on expression of specific regeneration-associated genes. Following a right facial nerve crush at the SMF, gonadectomized adult male rats were administered only ES, only TP, a combination of both, or left untreated. Real time RT-PCR analysis was used to assess fold changes in mRNA levels FMN at 0 h, 6 h, 1 d, 2 d, 7 d, and 21 d post-axotomy. The candidate genes analyzed included two tubulin isoforms

( $\alpha_1$ -tubulin and  $\beta_{II}$ -tubulin), GAP-43, BDNF, pituitary adenylate cyclase-activating peptide (PACAP), and neuritin (candidate plasticity-related gene 15). The two treatments were found to have differential effects on gene expression, with ES leading to early but transient upregulation and TP producing late but steady increases in mRNA levels. In comparison to individual treatments, the combinatorial treatment strategy had the most enhanced effects on the transcriptional program activated following injury.

## B. INTRODUCTION

Despite the robust ability of the peripheral nervous system to regenerate, functional and clinically relevant recovery following injury is often suboptimal. Delays in nerve regeneration may occur due to hindered axonal outgrowth across the site of injury, slow regeneration rate over long distances, inadequate reinnervation, or target atrophy (Valero-Cabre et al., 2004; Lee and Wolfe, 2000; Lundborg, 2000). A crush or transection injury switches a fully differentiated adult neuron into a growth mode, inducing a coordinated pattern of gene expression that underlies the intrinsic regenerative capacity of peripheral nerves. This growth program includes an increase in expression of transcription factors, cell-adhesion molecules, growth factors, cytokines, and structural components needed for axonal elongation (Makwana and Raivich, 2005). Therapeutic strategies that accelerate the onset or enhance the degree of this regenerative response may improve functional recovery outcome following injury.

Gonadal steroid hormones have significant trophic effects on the nervous system and play a neuroprotective role in conditions of injury or disease (Schumacher et al.,

1996; De Nicola, 1993; Jones, 1993a; Bialek et al., 2004; Tetzlaff et al., 2006; Fargo et al., 2008b). Over a course of several studies in the rodent facial nerve axotomy model, in which the nerve is crushed or transected at its exit from the SMF, our lab has shown that testosterone administration at the time of injury accelerates functional recovery and increases the rate of nerve regeneration (Tetzlaff et al., 2006; Kujawa and Jones, 1990; Kujawa et al., 1989; Kujawa et al., 1991). These neurotherapeutic effects of testosterone are mediated by its binding to the AR, abundantly found in the motor nuclei of brainstem and spinal cord neurons (Yu and McGinnis, 2001; Tetzlaff et al., 2007a; Kujawa et al., 1995). Molecular studies further demonstrate that testosterone administration following injury attenuates GFAP expression and increases levels of ribosomal RNA and mRNA levels of regeneration-associated genes such as  $\beta$ II-tubulin and GAP-43 (Kinderman and Jones, 1994; Jones and Oblinger, 1994; Jones et al., 1997a; Jones et al., 1997b). However, since receptor-mediated mechanisms require multiple steps for signal transduction, the therapeutic effects of testosterone may be delayed in comparison to therapies that can exert more immediate effects.

Multiple studies have shown that low frequency ES of the proximal nerve stump following injury promotes axonal regeneration. ES in the rat femoral and sciatic nerve injury models results in enhanced regeneration and improved reinnervation specificity (Al-Majed et al., 2000a; Vivo et al., 2008). Studies also report that ES accelerates the onset of functional recovery, increases axonal growth across the injury site, and improves neuronal survival following axotomy (Pockett and Gavin, 1985; Brushart et al., 2002; Morimoto et al., 2002). Although the mechanisms by which ES mediates its effects are



not fully known, it has been demonstrated that it influences the neuronal soma response, rapidly increasing expression of neurotrophin-4/5 and BDNF, as well as their receptor trkB (English et al., 2006; Al-Majed et al., 2000b). ES is also associated with increased expression of  $\alpha_1$ -tubulin and GAP-43 in motor and sensory nerve regeneration systems (Al-Majed et al., 2004; Geremia et al., 2007).

We have previously investigated the therapeutic potential of ES and TP in the extratemporal facial nerve injury model and found that the combinatorial treatment has additive effects on acceleration of functional recovery (Lal et al., 2008; Hetzler et al., 2008). To investigate the mechanism underlying the additive effects of gonadal steroids and ES, we used a radioisotopic labeling method to measure facial nerve outgrowth in Aim 1 and found that ES of the proximal nerve stump reduced the delay before sprout formation begins but failed to accelerate the regeneration rate. TP treatment, on the other hand, augmented the regeneration rate but had no effect on the sprouting delay. While each single treatment had differential effects, their combined treatment enhanced both of the regenerative properties, resulting in an accelerated rate and a reduced delay in sprouting. Therefore, the combinatorial treatment strategy may have a better therapeutic potential since ES may be used to initiate regeneration and testosterone to increase the rate of elongation over time.

Since a successful regenerative response is dependent upon expression of regeneration-associated genes, the objective of the current study (Aim 2) was to examine the effects of ES and TP on gene regulation. We hypothesized that treatment with ES or TP alone may upregulate different populations of genes and/or display temporal

differences in gene regulation, while their combined treatment may have additive effects. The number of genes that compose the regeneration program is immense and yet incomplete. Therefore, the present study characterized changes in expression of six genes in response to ES and TP. These genes were chosen as they have previously been shown to be upregulated in the FMN following injury. These include two members of the tubulin family ( $\alpha_1$ -tubulin and  $\beta_{II}$ -tubulin) that provide cytoskeletal support for the elongating axon, as well as GAP-43, a well-known regeneration-associated gene that regulates the growth cone during regeneration. The study also includes BDNF and PACAP, known for their multiple neurotrophic effects. Lastly, we analyzed expression of neuritin, originally identified as candidate plasticity-related gene 15, which enhances neurite extension *in vitro* and induces dendritic growth and axonal elaboration *in vitro* (Nedivi et al., 1998, Cantallops et al., 2000, Javaherian and Cline, 2005). Characteristics of the six candidate genes are outlined in Table 2. Following a facial nerve crush injury in adult rats, the effects of ES and/or TP on gene expression were assessed using real time RT-PCR analysis. The results demonstrate that the two treatments not only target different genes, but also that ES rapidly but transiently upregulated gene expression whereas TP had delayed but sustained effects in facial motoneurons.

## C. MATERIALS AND METHODS

### 1. ANIMALS & NERVE INJURY PARADIGM

Adult male Sprague-Dawley rats (~2 months old) were purchased from Harlan

**Table 2. Characteristics of candidate regeneration-associated genes**

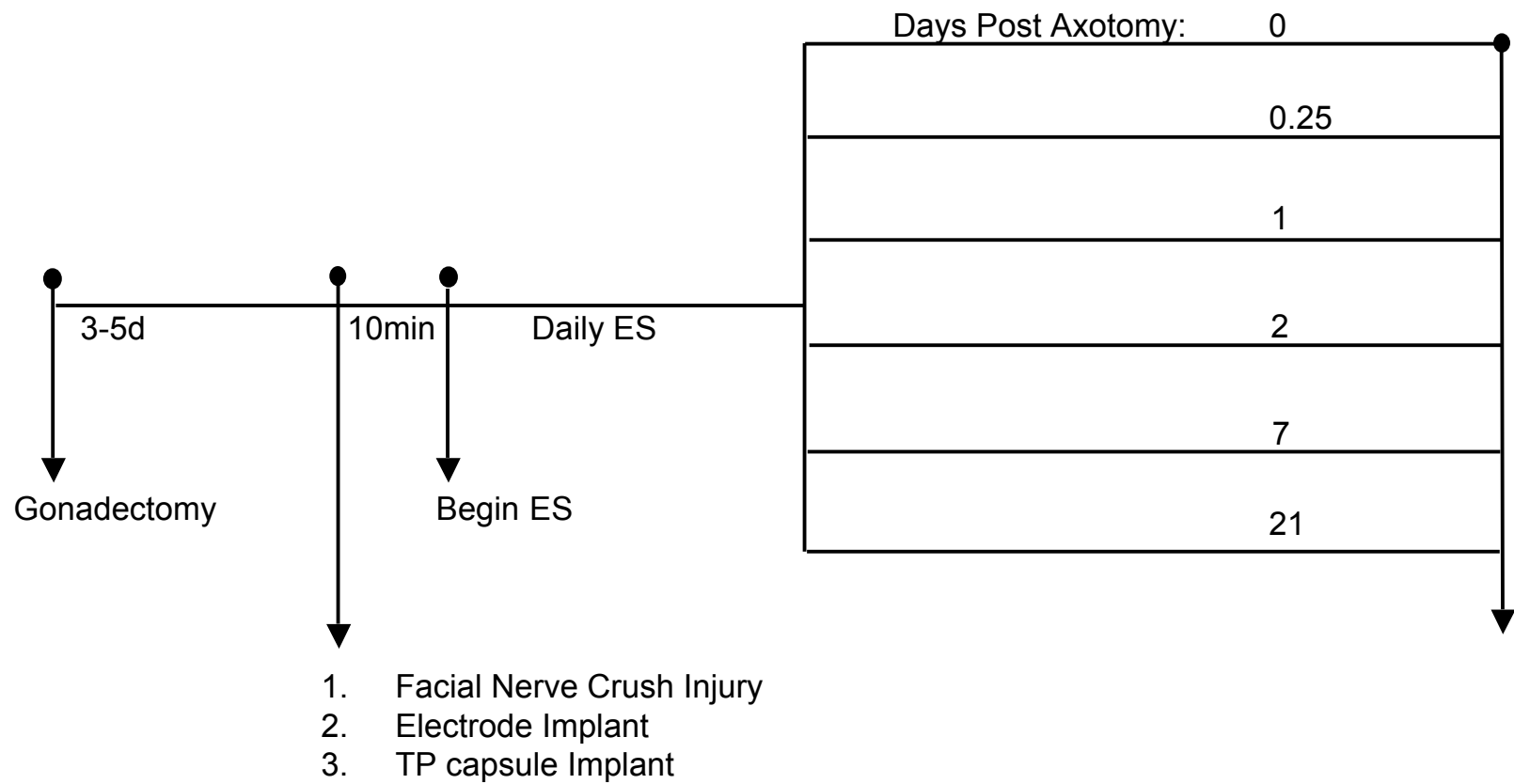
<b>Candidate Gene</b>	<b>Known Functions or Reported Effects</b>
$\alpha$ 1 Tubulin	Isotype of the tubulin family; Cytoskeletal protein that supports the regenerating axon and growth cone
$\beta$ II Tubulin	Isotype of the tubulin family; Cytoskeletal protein that supports the regenerating axon and growth cone
GAP-43	Regulates growth cone during nervous system development and regeneration; Increased expression increases sprouting and prevents growth cone collapse
BDNF	Neurotrophic factor; Reduces motoneuron loss after axotomy and reverses soma atrophy
PACAP	Neuropeptide with pleiotropic effects; Promotes neuritic outgrowth and/or cell survival in CNS and nerve regeneration in PNS; Interacts with neurotrophic factors
Neuritin	Membrane-anchored protein; Regulated by synaptic activity; Promotes dendritic and axonal growth <i>in vivo</i> and neurite outgrowth <i>in vitro</i> ; Regulated by neurotrophic factors and androgens

(Indianapolis, IN) and used for all experiments, as described in Chapter III. Three to five days prior to nerve injury, rats were anesthetized with isofluorane and castrated. For facial nerve axotomy, rats were anesthetized by intra-peritoneal injections of Ketamine (100mg/ml; 0.1ml/100g body weight) and Xylazine (20mg/ml; 0.025ml/100g body weight). The right facial nerve was crushed near its exit from the SMF, as described previously in Chapter III. Two successive 30-second crushes, on alternating sides, were done with fine jeweler's forceps to ensure a full crush.

The experimental design for this study is illustrated in Figure 8. Animals were divided into 4 experimental groups: [1] no treatment, animals receiving axotomy but no treatment, [2] ES only, animals receiving axotomy and ES treatment, [3] TP only, animals receiving axotomy and TP treatment, and [4] ES + TP, animals receiving axotomy and the combination of ES and TP treatments.

## 2. ELECTRICAL STIMULATION

A custom electrode apparatus constructed in our laboratory was implanted in all rats (refer to Chapter III for details). Two Teflon-coated wires, bared of insulation for 2-3mm, were soldered to two "male" connector pins in a connector strip. The connector assembly was cemented into a syringe base using dental acrylic. At the time of axotomy, the base of the syringe was sutured onto the paraspinal back muscles of rats. Wires were run through subcutaneously and sutured ~2mm proximal to the injury site (cathode) and ~3-5mm away from it (anode). The connector pins of rats were attached to leads of an isolated pulse stimulator, and rats were stimulated at a voltage at which they displayed a



**Figure 8: Experimental Design for Aim 2.** This timeline will be followed for all four experimental groups: no treatment, ES-only, TP-only, and ES plus TP.

right ear flutter. Starting immediately post-axotomy, rats were either stimulated with supramaximal pulses delivered at a frequency of 20Hz or sham stimulated for 30 min daily until sacrifice.

### 3. HORMONE ADMINISTRATION

Immediately following injury, two Silastic capsules (0.062 in. id x 0.095 in. od; 10-mm length), equilibrated in physiological saline and containing 100% crystalline TP (Sigma), were subcutaneously implanted in rats receiving hormone treatment. The dosage given has previously been shown to establish supraphysiological levels of systemic TP (Kujawa et al., 1989; Hetzler et al., 2008; Tanzer and Jones, 2004).

### 4. REAL-TIME RT-PCR

Rats were sacrificed by CO<sub>2</sub> asphyxiation at 0 h, 6 h, 1 d, 2 d, 7 d, or 21 d following facial nerve axotomy. Brains were removed and placed in a rat brain matrix on ice to provide support during sectioning. Using 2 razor blades spaced 1mm apart, sections were taken at the border of the pontine band and moving caudally. The precise rostral-caudal location of the facial motor nucleus and symmetry between the control and axotomized side was verified by identifying the internal genu of the facial nerve rostral to the site of section. On the coronal sections taken, 1mm concentric punches of the control and axotomized facial motor nuclei were collected for harvest. Tissue was homogenized with Lysing Matrix D and Solution D, and RNA was extracted, precipitated, and quantified on a Nano-Drop Spectrophotometer. DNase treatment and reverse

transcription on 100 ng of total RNA were done to obtain cDNA samples (refer to Chapter III for details).

The following rat-specific primers purchased from SuperArray Biosciences were used to amplify cDNA of candidate genes:  $\alpha$ 1 tubulin,  $\beta$ II Tubulin, GAP43, BDNF, PACAP, and neuritin. PCR amplification was carried out in triplication using SYBR Green PCR Mix. Cycling parameters were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles comprising 15 sec at 95°C and 60 sec at 60°C. After PCR amplification, the samples were heated from 60°C to 95°C to perform melt curve analysis and verify the specificity of the amplified products. Fold changes in mRNA levels on the axotomized side as compared to the control side were calculated using the  $2^{(-\Delta\Delta Ct)}$ -1 method, and fold changes were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

## 5. STATISTICAL ANALYSIS

Significant changes in mRNA levels among all four experimental groups were determined using a two-way ANOVA (factors = days post-axotomy and treatment), followed by a Newman-Keuls' multiple comparison post-hoc test at  $p < 0.05$ . However, for the ease of visually displaying the differences, three graphs were plotted for each gene analyzed, to compare the fold change in mRNA levels between the no treatment and each of the treatment groups (ES only, TP only, or ES plus TP).

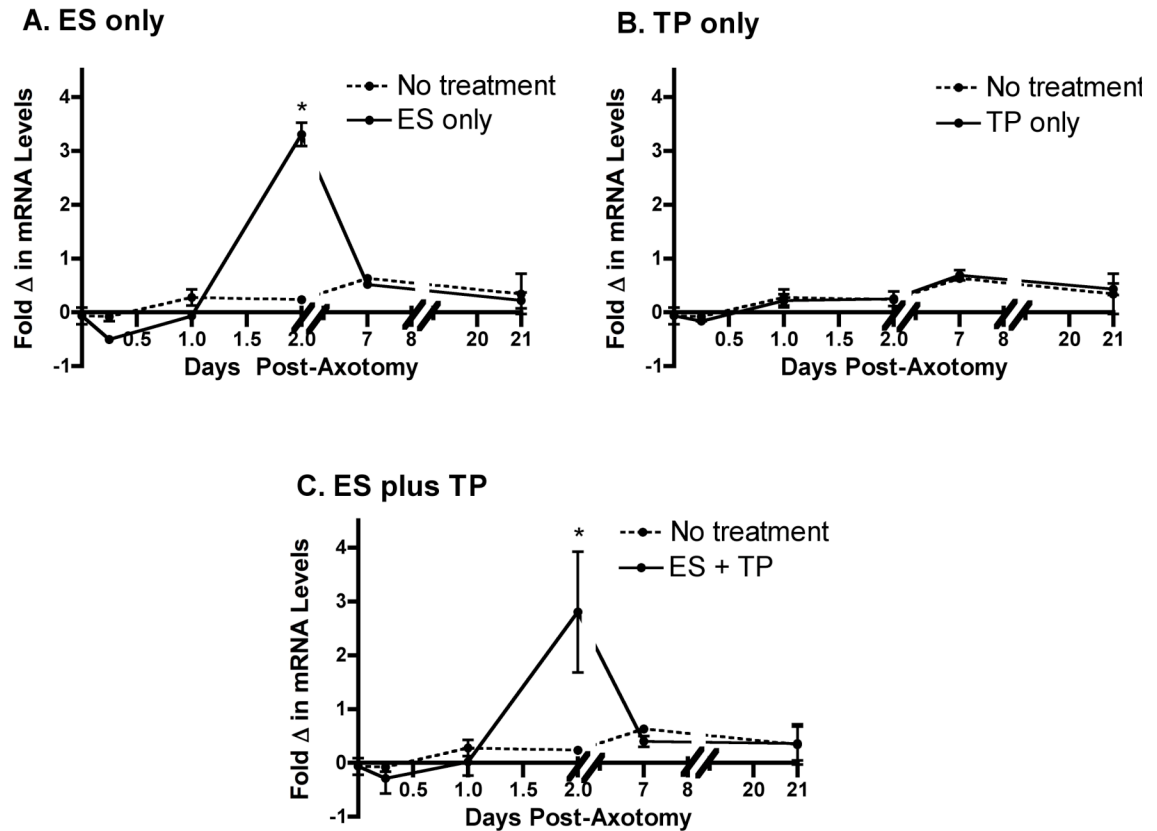
## D. RESULTS

### 1. EFFECTS OF ES AND TP ON EXPRESSION OF TUBULIN ISOFORMS

The gene expression pattern in the axotomized, untreated animals confirmed that injury alone significantly increased the fold change in  $\alpha_1$ -tubulin mRNA levels by 7 dpo ( $0.63 \pm 0.07$ ), as compared to baseline levels ( $-0.06 \pm 0.15$ ;  $p < 0.05$ ; Fig. 9). Figure 9A demonstrates that compared to no treatment, ES of the facial nerve significantly enhance  $\alpha_1$ -tubulin expression as early as 2 dpo ( $0.23 \pm 0.05$  vs.  $3.30 \pm 0.22$  fold change, respectively;  $p < 0.05$ ). By 7 dpo, the fold change in  $\alpha_1$ -tubulin mRNA levels in ES-treated animals decreased to values of untreated animals ( $0.52 \pm 0.06$  vs.  $0.63 \pm 0.07$ , respectively).

Systemic administration of TP did not alter  $\alpha_1$ -tubulin expression patterns relative to no treatment. Fold change in  $\alpha_1$ -tubulin mRNA levels in TP-treated animals was also highest 7 dpo ( $0.69 \pm 0.10$ ), as compared to  $0.63 \pm 0.07$  with no treatment (Fig. 9B). However, combining ES with TP more rapidly upregulated  $\alpha_1$ -tubulin expression, with the fold change in mRNA levels peaking to  $2.80 \pm 1.12$  at 2 dp0 ( $p < 0.05$  as compared to control; Fig. 9C). By 7 dpo,  $\alpha_1$ -tubulin expression in the combined treatment group returned to values of untreated animals ( $0.40 \pm 0.10$  vs.  $0.63 \pm 0.07$  fold change, respectively). This expression pattern of  $\alpha_1$ -tubulin in the combined treatment group was upregulated in a manner similar to the ES only group. Therefore, the data suggest that ES of the facial nerve leads to an earlier and significant increase in  $\alpha_1$ -tubulin expression, while TP has no supplementary effect.



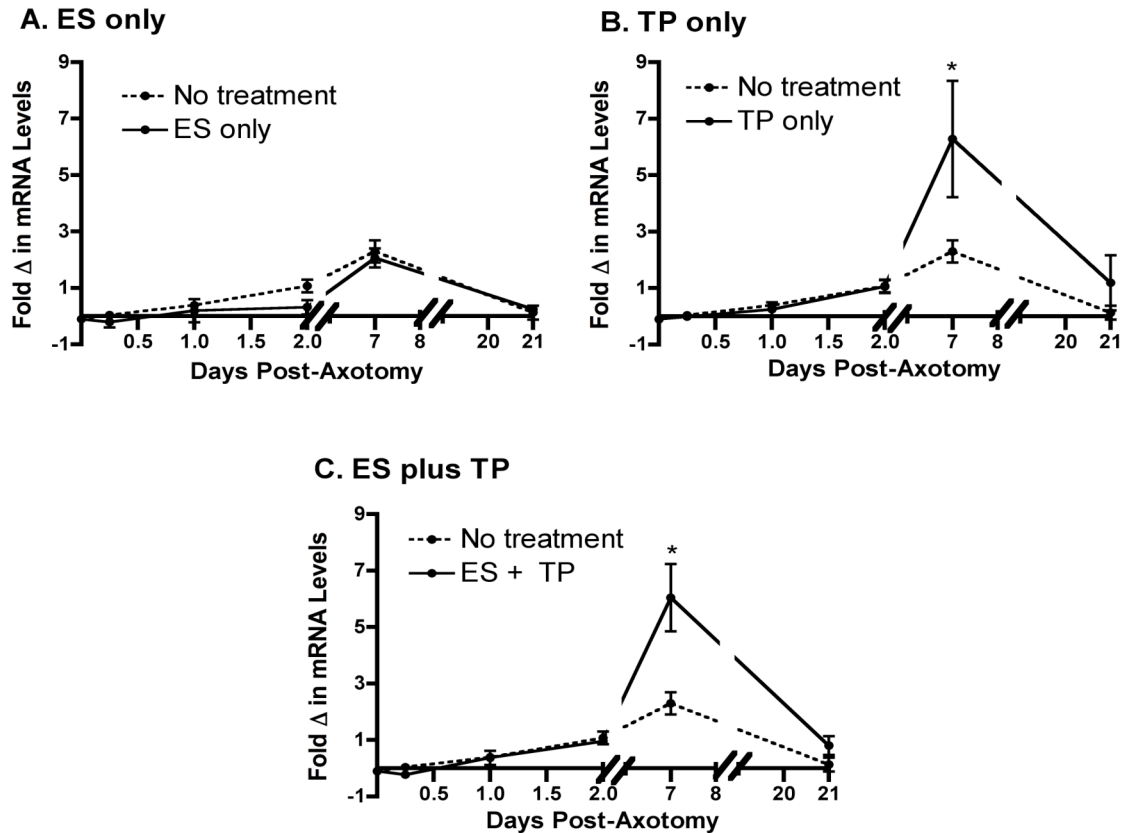


**Figure 9. Effects of ES and TP on expression of  $\alpha_1$ -tubulin following an extratemporal facial nerve crush injury in rats.** Shown is a time-course of fold changes in mRNA levels of  $\alpha_1$ -tubulin in the axotomized FMN relative to the control FMN, with post-operative survival times being 0h, 6h, 1d, 2d, 7d, and 21d. Following axotomy, untreated animals are compared to animals receiving only ES (A), only TP (B), or the combination of both (C). Within the untreated group,  $\alpha_1$ -tubulin is significantly higher 7d following injury, relative to the baseline levels at 0h ( $p < 0.05$ ). Asterisks are used to represent significant differences between different groups ( $p < 0.05$ ). Vertical lines represent standard error of the mean. For each experimental group,  $n = 3-6$  animals/time point.

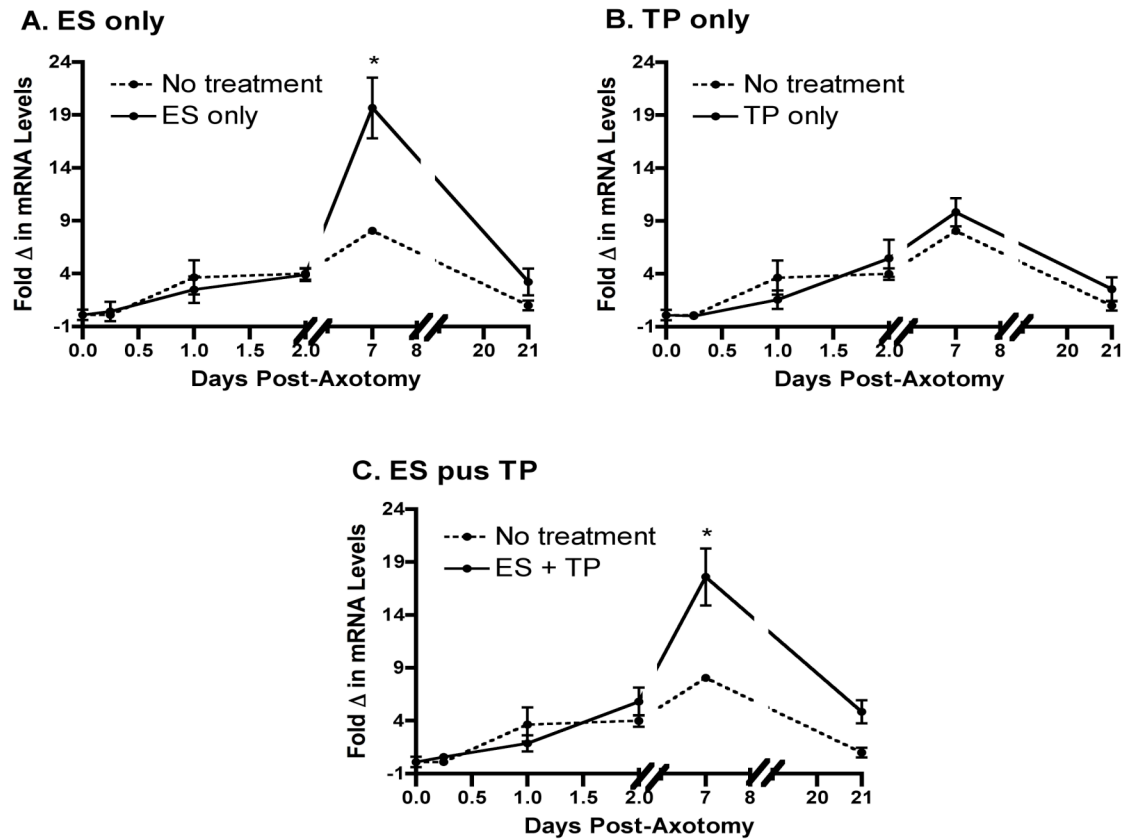
$\beta_{II}$ -tubulin expression was also upregulated in axotomized, untreated animals by a  $2.30 \pm 0.39$  fold change at 7 dpo, relative to baseline levels ( $0.08 \pm 0.08$ ;  $p < 0.05$ ; Fig. 10). However, the treatments produced an effect contrary to that seen with  $\alpha_1$ -tubulin. ES did not alter the timing or degree of upregulation in  $\beta_{II}$ -tubulin expression, since the highest fold change ( $2.47 \pm 0.34$ ) at 7 dpo was comparable to the no treatment group (Fig. 10A). Administration of TP, however, augmented the fold change in  $\beta_{II}$ -tubulin mRNA levels to  $6.28 \pm 2.06$  at 7 dpo, relative to no treatment ( $2.30 \pm 0.39$ ;  $p < 0.05$ ; Fig 10B). Although this increase in expression did not occur earlier, the magnitude of the change was nearly tripled. When the combination treatment of ES plus TP was given, the fold change in  $\beta_{II}$ -tubulin expression again increased to  $6.04 \pm 1.19$  at 7 dpo ( $p < 0.05$  relative to no treatment; Fig 10C). Therefore, systemic administration of TP augmented  $\beta_{II}$ -tubulin expression while ES had no additional effect.

## 2. EFFECTS OF ES AND TP ON EXPRESSION OF GAP-43

In axotomized untreated animals, the fold change in GAP-43 expression significantly increased at 2 dpo and peaked at 7 dpo ( $3.98 \pm 0.55$  and  $8.05 \pm 0.24$ , respectively;  $p < 0.05$  relative to baseline; Fig 11). Treatment with ES enhanced this change in GAP-43 expression by an additional ~10-fold, raising the peak to  $19.6 \pm 2.86$  at 7 dpo ( $p < 0.05$  as compared to control; Fig 11A). Administration of TP alone had no additional effect on GAP-43 expression, with the degree of fold change in mRNA levels being  $7.85 \pm 1.19$  at 7 dpo, similar to the  $8.05 \pm 0.24$  fold change observed in untreated animals (Fig 11B).



**Figure 10. Effects of ES and TP on expression of  $\beta_{II}$ -tubulin following an extratemporal facial nerve crush injury in rats.** Shown is a time-course of fold changes in mRNA levels of  $\beta_{II}$ -tubulin in the axotomized FMN relative to the control FMN, with post-operative survival times being 0h, 6h, 1d, 2d, 7d, and 21d. Following axotomy, untreated animals are compared to animals receiving only ES (A), only TP (B), or the combination of both (C). Within the untreated group,  $\beta_{II}$ -tubulin is significantly higher 7d following injury, relative to the baseline levels at 0h ( $p < 0.05$ ). *Asterisks* are used to represent significant differences between different groups ( $p < 0.05$ ). Vertical lines represent standard error of the mean. For each experimental group,  $n = 3-6$  animals/time point.



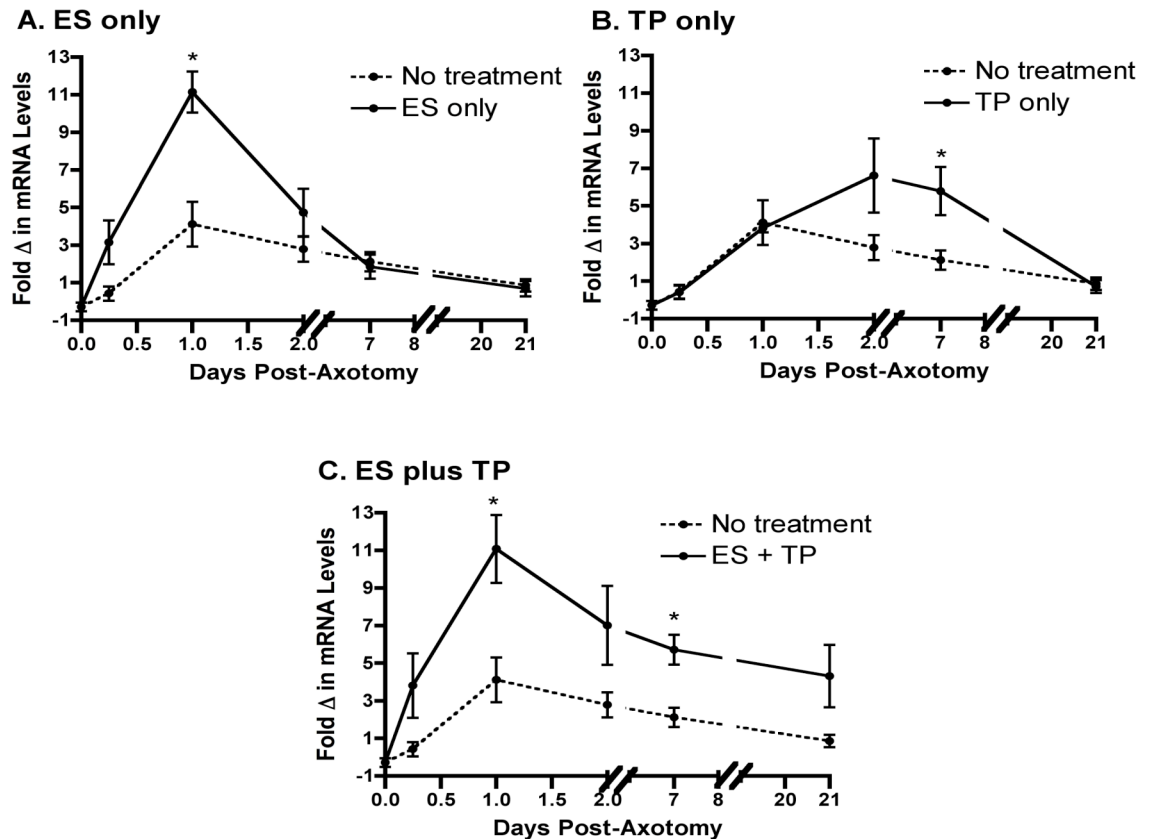
**Figure 11. Effects of ES and TP on expression of GAP-43 following an extratemporal facial nerve crush injury in rats.** Shown is a time-course of fold changes in mRNA levels of GAP-43 in the axotomized FMN relative to the control FMN, with post-operative survival times being 0h, 6h, 1d, 2d, 7d, and 21d. Following axotomy, untreated animals are compared to animals receiving only ES (A), only TP (B), or the combination of both (C). Within the untreated group, GAP-43 is significantly higher 2-7d following injury, relative to the baseline levels at 0h ( $p < 0.05$ ). *Asterisks* are used to represent significant differences between different groups ( $p < 0.05$ ). Vertical lines represent standard error of the mean. For each experimental group,  $n = 3-6$  animals/time point.

Treatment with the combination of ES plus TP upregulated GAP-43 expression by a  $17.6 \pm 2.7$  fold change at 7 dpo (Fig 11C). This increase in the combined treatment group was significantly higher than that seen in untreated animals ( $8.05 \pm 0.24$  fold change;  $p < 0.05$ ) but similar to that seen in the ES only group ( $19.6 \pm 2.86$  fold change; Fig 11A). Administration of ES did not accelerate the time at which GAP-43 expression peaked (Fig 11A and C). Nonetheless, ES of the proximal nerve stump did significantly increase the fold change in GAP-43 mRNA levels while TP treatment had no added effect on GAP-43 expression.

### 3. EFFECTS OF ES AND TP ON EXPRESSION OF BDNF

In axotomized, untreated animals, BDNF expression was significantly upregulated to a  $5.15 \pm 1.33$  fold change at 1 dpo, relative to baseline ( $p < 0.05$ ; Fig 12). At 1 dpo, treatment with ES led to an additional 5-fold upregulation in BDNF expression, with levels increasing to  $11.14 \pm 1.14$ -fold ( $p < 0.05$  as compared to control; Fig 12A). However, in both untreated and ES-treated animals, BDNF mRNA levels began to decline by 2 dpo.

At 1 dpo, untreated and TP-treated animals displayed a similar fold change in BDNF mRNA levels ( $5.15 \pm 1.33$  and  $3.82 \pm 0.22$ , respectively; Fig 12B). At 2 and 7 dpo, however, TP treatment maintained BDNF expression ( $6.28 \pm 2.11$  and  $5.79 \pm 1.28$  fold change, respectively), while levels declined to  $2.78 \pm 0.67$  and  $2.12 \pm 0.61$  fold change, respectively, in untreated animals ( $p < 0.05$ ; Fig 12B). The combinatorial treatment displayed both the early effects of ES and late effects of TP (Fig 12C). At 1



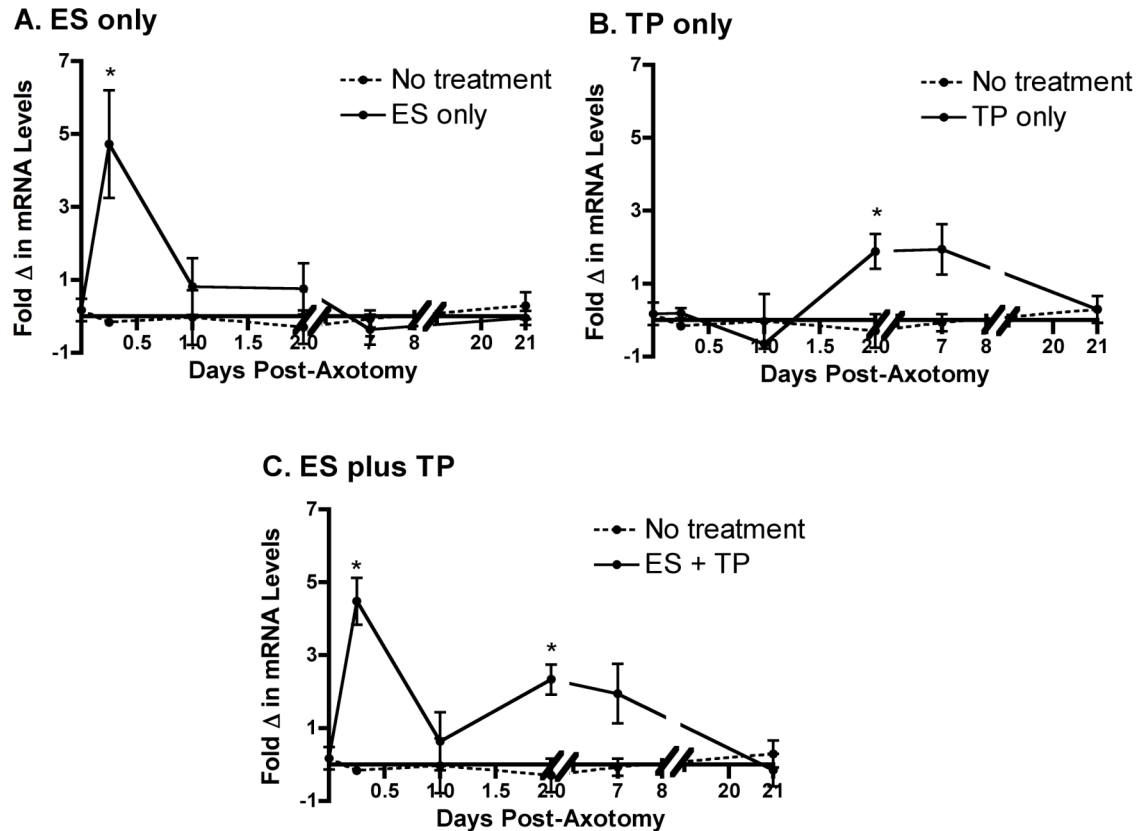
**Figure 12. Effects of ES and TP on expression of BDNF following an extratemporal facial nerve crush injury in rats.** Shown is a time-course of fold changes in mRNA levels of BDNF in the axotomized FMN relative to the control FMN, with post-operative survival times being 0h, 6h, 1d, 2d, 7d, and 21d. Following axotomy, untreated animals are compared to animals receiving only ES (A), only TP (B), or the combination of both (C). Within the untreated group, BDNF is significantly higher 1d following injury, relative to the baseline levels at 0h ( $p < 0.05$ ). Asterisks are used to represent significant differences between different groups ( $p < 0.05$ ). Vertical lines represent standard error of the mean. For each experimental group,  $n = 3-6$  animals/time point.

dpo, an additional 6-fold increase in BDNF levels occurred with the ES plus TP treatment ( $11.08 \pm 1.80$ ), relative to no treatment ( $5.15 \pm 1.33$ ;  $p < 0.05$ ). This increase was sustained until 7d post-axotomy, when the fold change in mRNA levels was at  $5.72 \pm 0.79$  in animals receiving the combined treatment and  $2.12 \pm 0.61$  in untreated animals ( $p < 0.05$ ). Together, these data suggest that ES rapidly increases BDNF expression for a transient period, whereas TP induces a late but long-term increase. In the combined treatment group, a temporally additive effect of both treatments was seen, with an immediate yet sustained upregulation of BDNF.

#### 4. EFFECTS OF ES AND TP ON EXPRESSION OF NEURITIN

Neuritin mRNA levels were not upregulated in axotomized, untreated animals at any time point post-axotomy (Fig 13). However, administration of ES significantly increased neuritin expression by a  $4.73 \pm 1.48$  fold change just 6 hours post-operative (hpo), relative to no treatment ( $-0.16 \pm 0.09$  fold change;  $p < 0.05$ ; Fig 13A). Following the rapid increase with ES, neuritin mRNA levels quickly returned to baseline by 1 dpo ( $-0.03 \pm 0.75$  fold change). Systemic TP treatment also upregulated neuritin expression. However, the effects were seen later at 2 dpo, when fold change in neuritin mRNA levels was  $1.89 \pm 0.47$  in TP-treated animals and  $-0.07 \pm 0.24$  in untreated ( $p < 0.05$ ; Fig 13B). In TP-treated animals, neuritin mRNA levels remained elevated at a fold change of  $1.94 \pm 0.69$  up till 7 dpo.

Early and late effects on neuritin upregulation were observed in animals receiving both ES and TP. With the combined treatment, neuritin mRNA levels rapidly



**Figure 13. Effects of ES and TP on expression of neuritin following an extratemporal facial nerve crush injury in rats.** Shown is a time-course of fold changes in mRNA levels of neuritin in the axotomized FMN relative to the control FMN, with post-operative survival times being 0h, 6h, 1d, 2d, 7d, and 21d. Following axotomy, untreated animals are compared to animals receiving only ES (A), only TP (B), or the combination of both (C). Within the untreated group, neuritin expression did not significantly increase at any time point, relative to the baseline levels at 0h ( $p < 0.05$ ). Asterisks are used to represent significant differences between different groups ( $p < 0.05$ ). Vertical lines represent standard error of the mean. For each experimental group,  $n = 3-6$  animals/time point.

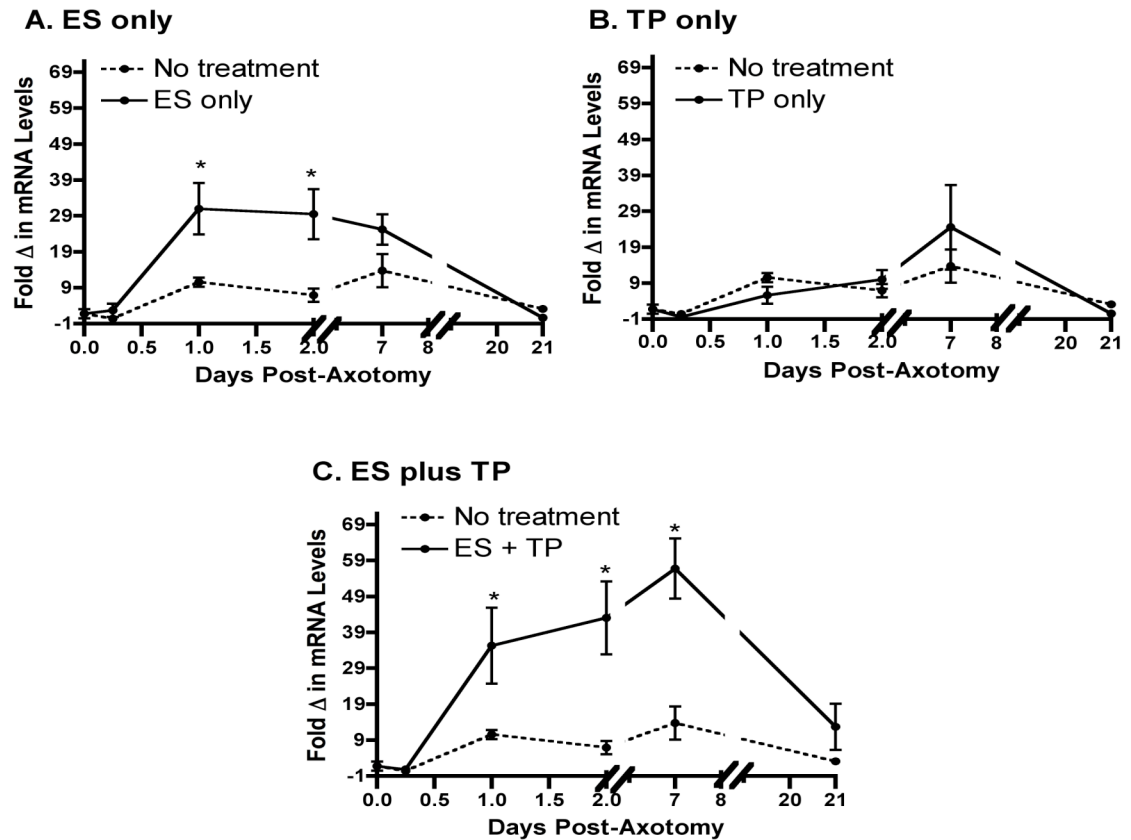


increased to a  $4.48 \pm 0.64$  fold change at 6 hpo, relative to no treatment ( $-0.16 \pm 0.09$ ;  $p < 0.05$ ; Fig 13C). Furthermore, neuritin levels remained elevated at a  $2.33 \pm 0.41$  fold change at 2 dpo ( $p < 0.05$  relative to no treatment). Like treatment with TP alone, the fold change in neuritin expression in the combined treatment group was also sustained until 7 dpo ( $1.94 \pm 0.82$ ). Therefore, administration of ES and TP together led to an immediate and prolonged upregulation of neuritin expression.

## 5. EFFECTS OF ES AND TP ON EXPRESSION OF PACAP

In axotomized, untreated animals, PACAP expression significantly increased to a  $10.56 \pm 1.25$  fold change at 1 dpo ( $p < 0.05$  relative to baseline; Fig 14). Furthermore, PACAP mRNA levels remained elevated until 7 dpo ( $13.76 \pm 4.63$  fold change). In animals receiving ES, the fold change in PACAP expression tripled to  $30.98 \pm 7.13$  at 1 dpo and  $29.50 \pm 6.98$  at 2 dpo, while the fold change in untreated animals was  $10.56 \pm 1.25$  at 1 dpo and  $6.52 \pm 2.51$  at 2d post-axotomy ( $p < 0.05$ ; Fig 14A). By 7 dpo, PACAP expression in animals receiving ES returned to levels of untreated animals ( $25.23 \pm 4.24$  and  $13.76 \pm 4.63$ , respectively).

Unlike ES, TP treatment had no additional effect on PACAP expression relative to no treatment. Fold change in PACAP mRNA levels remained elevated in TP-treated animals from 1 dpo ( $5.66 \pm 2.35$ ) to 7 dpo ( $24.56 \pm 11.78$ ), but there was no significant increase relative to untreated animals (Fig 14B). Combining TP with ES, however, had a dramatic effect on PACAP expression. The combined treatment led to a significant upregulation in PACAP expression, as the fold change in mRNA levels was  $27.45 \pm$



**Figure 14. Effects of ES and TP on expression of PACAP following an extratemporal facial nerve crush injury in rats.** Shown is a time-course of fold changes in mRNA levels of neuritin PACAP in the axotomized FMN relative to the control FMN, with post-operative survival times being 0h, 6h, 1d, 2d, 7d, and 21d. Following axotomy, untreated animals are compared to animals receiving only ES (A), only TP (B), or the combination of both (C). Within the untreated group, PACAP expression is significantly higher 2-7d post injury, relative to the baseline levels at 0h ( $p < 0.05$ ). Asterisks are used to represent significant differences between different groups ( $p < 0.05$ ). Vertical lines represent standard error of the mean. For each experimental group,  $n = 3-6$  animals/time point.

10.05 at 1 dpo and  $35.27 \pm 10.57$  at 2 dpo ( $p < 0.05$  relative to no treatment; Fig 14C).

However, at 7 dpo, there was a further increase in PACAP expression by  $56.72 \pm 8.37$ -fold, a change double than that seen with ES alone ( $25.23 \pm 4.24$ ) or TP alone ( $24.56 \pm 11.78$ ). Therefore, treatment with both ES and TP led to an additive effect on PACAP expression.

## E. DISCUSSION

Our previous studies have shown that administration of daily, low frequency ES accelerates the onset of functional recovery and reduces the initial delay in sprout formation but fails to increase the rate of axonal regeneration (Lal et al., 2008; Chapter IV). TP treatment, in contrast, accelerates the overall regeneration rate without affecting the sprouting delay. The combinatorial treatment of daily ES plus systemic TP was found to enhance functional recovery and facial nerve regenerative properties more than the use of either treatment alone (Hetzler et al., 2008; Chapter IV). In this study, we characterized the molecular effects of ES and TP, and determined that the two treatments enhance gene expression in distinct patterns when administered alone and have combined upregulatory effects when administered together.

Axonal elongation requires reconstruction of the microtubule cytoskeleton, composed of heterodimers of  $\alpha$  and  $\beta$  tubulin. Of the five different  $\alpha$ -tubulin isotypes and the five different  $\beta$ -tubulin isotypes expressed in the mammalian brain, axotomy upregulates expression of  $\alpha_1$ ,  $\beta_{II}$ , and  $\beta_{III}$  forms of tubulin (Hoffman and Cleveland, 1988; Miller et al., 1989; Moskowitz and Oblinger, 1995; Wong and Oblinger, 1990).

The present study confirmed that the mRNA levels of  $\alpha_1$ - and  $\beta_{II}$ -tubulin are upregulated following a facial nerve crush axotomy in rats. Our previous studies in the hamster facial motoneuron and the rat sciatic motoneuron systems have demonstrated that systemic administration of TP selectively upregulates expression of  $\beta_{II}$ -tubulin but not  $\alpha_1$ - or  $\beta_{III}$ -tubulin (Jones and Oblinger, 1994; Brown et al., 1999). Similarly, in the current study, we found that TP differentially upregulated mRNA levels of  $\beta_{II}$ -tubulin, but not  $\alpha_1$ -tubulin, in a time course similar to previous work. It is also important to note that although TP tripled the magnitude of  $\beta_{II}$ -tubulin fold change in the FMN, it did not induce an earlier increase in  $\beta_{II}$ -tubulin expression relative to no treatment. In contrast, ES had no additional effect on  $\beta_{II}$ -tubulin expression. Instead, it not only enhanced expression of  $\alpha_1$ -tubulin but also causes the upregulation to peak earlier as compared to axotomy without treatment. Al-Majed et al. (2004) have reported a similar increase in expression of  $\alpha_1$ -tubulin with 1 h ES of the rat femoral nerve following nerve transection and repair.

A potential mechanism by which ES mediates its effects is through the use of calcium and cAMP as second messengers (Brushart et al., 2002; Al-Majed et al., 2000a; Kocsis et al., 1994). Electrical activity is known to increase  $\text{Ca}^{2+}$  influx, which in turn has been coupled to changes in gene expression (Finkbeiner and Greenberg, 1998).  $\text{Ca}^{2+}$  influx also activates  $\text{Ca}^{2+}$ -dependent adenylate cyclase to increase intracellular levels of cAMP (Hempel et al., 1996; Shen et al., 1999). Previous studies have shown that cAMP itself can enhance axonal regeneration *in vivo* (Neumann et al., 2002; Gershenbaum and Roisen, 1980, Kilmer and Carlsen, 1984). Moreover, injecting dibutyryl cAMP, a

membrane-permeable analogue of cAMP, in the dorsal root ganglia increases levels of  $\alpha_1$ - and  $\beta_{III}$ -tubulin (Han et al., 2004). Activation of cAMP-dependent signaling pathways in primary cultures of guinea pig enteric ganglia also increases mRNA levels of  $\alpha_1$ -tubulin (Simeone et al., 1994). Although the effects of cAMP on  $\beta_{II}$ -tubulin expression have not been reported, the specific upregulation of  $\alpha_1$ -tubulin expression following treatment with ES may be due to cAMP-mediated effects.

Although it is unclear why ES and TP upregulate different isoforms of tubulin, there are several possible explanations. While there is 40% sequence conservation between  $\alpha$  and  $\beta$  forms of tubulin, the two can diversify further based upon their post-translational modifications, such as acetylation, phosphorylation, detyrosylation, polyglutamylation, and polyglycylation (Ludueno, 1998). The two isoforms may also differ in their interactions with microtubule-associated proteins. Such properties may affect the stability, dynamics, and motility of different  $\alpha\beta$  heterodimers, allowing them to impart unique properties to the cytoskeleton. The enhanced effect of ES on  $\alpha_1$ -tubulin expression may have some relation to it also reducing the delay before sprout formation begins and increasing levels of intracellular messengers that specifically activate transcription of the  $\alpha_1$ -tubulin, but not the  $\beta_{II}$ -tubulin, gene. On the other hand, TP-mediated increase in  $\beta_{II}$ -tubulin may be due to there being steroid response elements on the  $\beta_{II}$ -tubulin, but not the  $\alpha_1$ -tubulin, gene. An increase in  $\beta_{II}$ -tubulin may also be correlated to an increase in axonal elongation rate, a property enhanced by TP but not ES. ES and TP appear to be working through distinct mechanisms to specifically alter expression of either  $\alpha_1$ -tubulin or  $\beta_{II}$ -tubulin. The combinatorial treatment of ES plus TP

upregulates both  $\alpha_1$ - and  $\beta_{II}$ -tubulin, and thus may provide regenerating axons with the functional benefits of both isoforms.

Regulation of microtubule assembly and actin polymerization at nerve endings are crucial for axon elongation and synaptogenesis. GAP-43 is a well-known regeneration-associated gene implicated in this process during development and regeneration (Chong et al., 1992; Aigner et al., 1995). Transgenic mice overexpressing GAP-43 demonstrate spontaneous formation of new synapses and enhanced sprouting after injury, whereas a loss in function of the GAP-43 gene disrupts axonal pathfinding (Aigner et al., 1995; Strittmatter et al., 1995). In accordance with existing literature, current results demonstrate that a facial nerve crush, without any treatment, increased GAP-43 expression. Gonadal steroids have been shown to modulate expression of GAP-43 in the rat central nervous system (Shughrue and Dorsa, 1993a; Shughrue and Dorsa, 1993b; Lustig et al., 1991). In a previous study using *in situ* hybridization, our lab found that administration of systemic TP prevented the transient decline in GAP-43 mRNA levels seen in untreated hamsters 7 d following facial nerve transection (Jones et al., 1997a). The present study in rats slightly differs in that it found no dip in GAP-43 expression at 7d post-injury in the absence of treatment and thus no additional effects of TP.

ES of the transected and sutured rat femoral nerve for 1 h has been shown to accelerate the increase in GAP-43 expression (Al-Majed et al., 2004). Although our results demonstrate that 30 min of daily ES treatment also amplified GAP-43 expression, there are some differences. In our facial nerve injury model, we did not find that ES upregulated GAP-43 expression earlier than injury alone. Instead, the peak in GAP-43

expression occurs at the same time point following injury with or without ES treatment. The slight discrepancy in results between our study and the study by Al-Majed et al. (2004) may be due to differences in the molecular techniques used to quantify fold change (RT-PCR vs. in situ hybridization) and/or the nerve injury paradigms (facial nerve crush vs. femoral nerve transection and repair). Nevertheless, the current finding that ES enhances GAP-43 expression lends insight into its potential mechanism of therapeutic action and how it may be different from that of TP. The combinatorial treatment of ES plus TP upregulated GAP-43 expression similar to ES alone, further suggesting that ES enhances GAP-43 expression while TP has no supplementary effect.

Motoneurons can respond to neurotrophins secreted by non-neuronal cells or by autocrine signaling. Synthesis of BDNF and its receptor, trkB, increases in several motoneuron systems, including the facial nerve, following axonal injury (Meyer et al., 1992; Funakoshi et al., 1993; Piehl et al., 1994; Kobayashi et al., 1997). BDNF promotes motoneuron survival, reverses soma atrophy, and induces axonal outgrowth *in vivo* (Yan et al., 1992; Novikov et al., 1997; Novikova et al., 1997; Novikov et al., 1995; Kishino et al., 1997). There are several lines of evidence supporting the effects of gonadal steroids on BDNF expression. Gonadal steroids have been shown to regulate BDNF and trkB levels in the central and peripheral nervous systems (Ottem et al., 2007; Osborne et al., 2007; Sohrabji et al., 1995; Solum and Handa, 2002; Jezierski and Sohrabji, 2001). Plasma BDNF levels have also been positively correlated with endogenous levels of gonadal steroids (Begliuomini et al., 2007). The present study found that TP treatment

prolonged BDNF upregulation and maintained high mRNA levels during later phases of regeneration

Since various studies demonstrate that membrane depolarization also induces an increase in BDNF levels, electrical activity may play an important role in regulating BDNF expression (Zafra et al., 1990; Patterson et al., 1992). One hour ES of the rat femoral nerve has been shown to rapidly upregulate BDNF and *trkB* expression immediately following transection and surgical repair (Al-Majed et al., 2000b). Our results also demonstrate that ES immediately augmented the fold change in BDNF upregulation, but that the effects were short-termed. Combining ES with TP led to temporally additive effects: it produced the rapid increase in BDNF expression, as seen with ES alone, and it maintained the increase for a longer time course post-axotomy, as seen with TP alone.

Neuritin was originally identified as a candidate plasticity-related gene whose expression is regulated by neuronal activity (Nedivi et al., 1998; Naeve et al., 1997). It is a small, highly conserved, extracellular protein that is attached to the extracellular membrane via a glycosylphosphatidylinositol anchor. Neuritin promotes neurite outgrowth, enhances dendritic and axonal arborization, and plays a role in synapse maturation (Nedivi et al., 1998; Cantallops et al., 2000; Javaherian and Cline, 2005). Following spinal cord injury in rats, neuritin levels are initially downregulated for 24 h and then strongly re-induced during the following 2 wk (Di Giovanni et al., 2005). Neuritin expression also increases 0-6 h following ischemia and reperfusion in rats (Rickhag et al., 2007). The current study did not detect a significant increase in neuritin



mRNA levels over a course of 0 h to 21 d following a facial nerve crush, without treatment. Our previous study in hamsters also reported no change in neuritin expression in axotomized, untreated animals but found an increase upon administration of TP. Silencing neuritin has also been shown to abolish androgenic effects on neurite outgrowth *in vitro*, suggesting that neuritin is a downstream effector of androgens (Marron et al., 2005). The current results support these previous finding by demonstrating that TP administration enhances and steadily maintains neuritin expression.

Treatment with ES also enhanced neuritin expression, however, the effects were more immediate and transient as compared to TP treatment. Since membrane depolarization is known to modulate neuritin (Naeve et al., 1997), ES of the proximal nerve stump may alter electrical activity in a way that leads to early transcriptional changes in neuritin. Combining ES with TP led to both the early effects of ES and late effects of TP. Therefore, a combinatorial treatment strategy may be beneficial in that ES may immediately increase synthesis of immediate early genes (i.e. neurotrophic factors and neuritin) until the effects of testosterone come in to play and preserve high levels of gene expression over an extended time course.

Peripheral nerve axotomy has been shown to upregulate expression of PACAP in sensory neurons of the dorsal root ganglia and the mesencephalic trigeminal nucleus, the sympathetic neurons of the superior cervical ganglia, and the facial motor nucleus (Zhang et al., 1996; Zhang et al., 1995; Larsen et al., 1997; Moller et al., 1997; Zhou et al., 1999). PACAP promotes neurite outgrowth and cell survival, has anti-inflammatory effects, and is neuroprotective in conditions of injury such as ischemia, trauma,

neurodegeneration, and axotomy (Somogyvari-Vigh and Reglodi, 2004). In the present study, PACAP expression increased substantially following axotomy alone, without any treatment. While TP administration has no additional effect on PACAP expression, treatment with ES tripled the fold change soon after axotomy. Interestingly, combining ES with TP had additive effects on PACAP expression, suggesting that TP may potentiate the mechanisms used by ES to enhance PACAP expression.

A successful regenerative response following peripheral nerve injury requires coordinated expression of neurotrophic factors, growth-associated proteins, and structural components. Characterizing the effects of potential treatments on gene expression is beneficial to understanding their underlying mechanisms of improving axonal regeneration and functional recovery. The finding that ES and TP not only target different genes but also differ in their timing and duration of effects implies that they work through different mechanisms. While ES may accelerate the onset of regeneration and immediately upregulate gene expression, the effects may be transient. Testosterone-mediated effects may require a longer lag time, but the changes in gene expression may be sustained, leading to an overall increase in rate of regeneration. Since the two treatments appear to be working through different mechanisms, the use of a combinatorial treatment strategy would maximize therapeutic effects and thus warrants further investigation for clinical translation.

**CHAPTER VI**  
**COMPARISON OF EXTRATEMPORAL AND INTRATEMPORAL**  
**FACIAL NERVE INJURY MODELS**

**A. ABSTRACT**

The facial nerve is the most commonly injured cranial nerve, and injury results in significant functional and emotional disturbances. Due to the nerve's complex anatomical course, injuries can occur at different proximities from the FMN and lead to varying outcomes. The purpose of the third aim of this dissertation was to compare functional recovery and motor nerve conduction following a distal, extratemporal crush injury of the facial nerve at its exit from the SMF to a more proximal, intratemporal crush injury of the nerve during its course in the temporal bone. Adult male rats were divided into four experimental groups: extratemporal crush, extratemporal sham-operated, intratemporal crush, and intratemporal sham-operated. Animals were observed daily for return of facial nerve function, including the eyeblink reflex and vibrissae orientation and movement. Motor nerve conduction studies were done weekly to quantify the changes in peak amplitude and latency of evoked response. Assessment of facial motoneuron survival was done at 4 and 8 weeks post-operative (wpo). Rats receiving the extratemporal facial nerve injury recovered full facial function by ~2 wpo and normal peak amplitude and latency recordings by 4 wpo and demonstrated no significant

neuronal loss post-mortem. In comparison, rats receiving the intratemporal facial nerve injury failed to reach complete functional recovery and peak amplitude of evoked response remained ~70% below normal at the end of 8 wpo, and they demonstrated a ~15% cell loss. The findings indicate that the location of facial nerve injury strongly influences the recovery and regeneration outcome.

## B. INTRODUCTION

Facial nerve lesions are common clinical conditions that result in significant distortion of the face and severe emotional and psychological disturbances in patients. Additional complications of facial nerve injury often include synkinesis, eye infections, corneal ulceration, drooling, and speech difficulties (Mavrikakis, 2008). Although facial function may return completely in some patients, others may demonstrate delayed recovery or residual deficits. Unsatisfactory recovery may be due to significant neuronal loss, inadequate reinnervation, misdirection of regenerating axons, or denervation-induced target atrophy.

The susceptibility of the facial nerve to injury arises from its complex anatomical course from the brainstem to its muscles of innervation. As the nerve travels intracranially, it may be injured through compression by tumors in the cerebellopontine angle (Danner, 2008). When the facial nerve enters the temporal bone, it travels an intricate course confined within a prolonged canal, which in some cases is not much greater in diameter than the nerve itself. In humans, this 30-mm course of the facial nerve within the temporal bone, the longest interosseous course of any cranial nerve, renders the nerve vulnerable to swelling and compression (Mavrikakis, 2008). Bell's

palsy, categorized as idiopathic, is the most common cause of facial paralysis, and believed to result from inflammation of the facial nerve during its course in the temporal bone, leading to compression and possibly ischemia and demyelination (Tiemstra and Khatkhate, 2007). Other causes of intratemporal facial nerve injuries include temporal bone fractures, iatrogenic injuries, infections and compression by neoplasms (Mavrikakis, 2008). As the nerve exits the skull at the SMF, it courses through the parotid gland to branch into five main branches that innervate the facial muscles. There it may become injured due to lacerations or complications arising from compression by or removal of parotid gland tumors (Danner, 2008). The site of facial nerve injury influences the severity of facial paresis and the recovery outcome.

Current treatments for facial nerve paralysis following transection injuries include direct anastomosis or cable grafting (Kumar et al., 2006). Diagnosis of Bell's palsy is usually followed up with a course of corticosteroids and anti-virals, but their efficacy is still controversial (Gilden, 2004). For some patients, recovery from facial paralysis begins within a few weeks and is completed within a few months to a year. However, this is not always the case; many patients have incomplete recovery and long-term sequelae from the paralysis (Mavrikakis, 2008). Several factors influence the outcome of regeneration, including the type or severity of injury, time between injury and treatment, age of patient, and location of the injury. Therefore, animal models that mimic the common facial nerve injuries seen in the clinic need to be developed so that potential therapies can be suitably investigated.

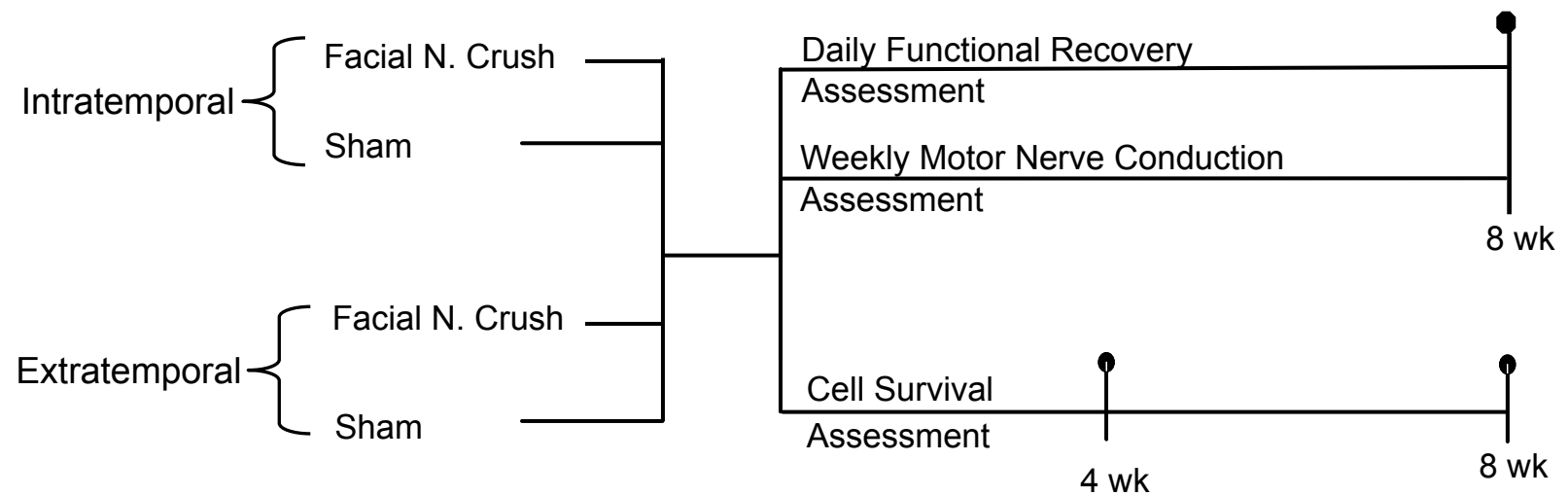
Currently, existing animal models of facial nerve injury include intracranial transection and extratemporal transection and crush of the facial nerve (Mattsson, 1999;

Jones, 2001). Thus far, an animal model of intratemporal facial nerve injury has not been characterized even though a majority of facial nerve lesions occur during its course through the temporal bone. Therefore, the third aim of this dissertation aimed to develop a more clinically relevant intratemporal model of facial nerve injury and compare it to the well-established extratemporal facial nerve injury model. Also, many insults to the facial nerve result from a stretching or compression of the nerve rather than its transection. Therefore, to more closely resemble such a clinical picture, this study used crush injury, in which the neural sheath is left intact while the axons are damaged. Our findings demonstrated significant differences in recovery of facial function, motor nerve conductance, and motoneuron survival between the extratemporal and intratemporal models of facial nerve injury.

## B. MATERIALS AND METHODS

### 1. ANIMALS

Adult male Sprague-Dawley rats (~2 months old) were purchased from Harlan (Indianapolis, IN) and used for all experiments, as described in Chapter III. For facial nerve crush injuries and weekly electromyographic (EMG) recordings, rats were anesthetized by intra-peritoneal injections of Ketamine (100mg/ml; 0.1ml/100g body weight) and Xylazine (20mg/ml; 0.025ml/100g body weight). The experimental design for this study is illustrated in Figure 15. Animals were divided into four experimental groups based on the type of facial nerve injury administered: 1) extratemporal crush, 2)



**Figure 15: Experimental Design for Aim 3.** This timeline will be followed to compare the two models of facial nerve injury, extratemporal and intratemporal.

extratemporal sham-operated, 3) intratemporal crush, and 4) intratemporal sham-operated.

## 2. EXTRATEMPORAL FACIAL NERVE CRUSH INJURY

A post-auricular incision was made, and the right facial nerve was exposed at its exit from the SMF (refer to Chapter III for details). Approximately 1 mm distal to the SMF, before branching of the main trunk, the nerve was crushed with a hemostat for 1 min. The standard crush performed ensured that all nerve fibers were crushed while the axonal sheath remained intact. For the extratemporal sham injury group, the right facial nerve was exposed but left uncrushed. In all animals, the left facial nerve was left intact to serve as an internal control. The post-operative survival time was 4 weeks, at which point the animals were sacrificed by CO<sub>2</sub> asphyxiation

## 3. INTRATEMPORAL FACIAL NERVE CRUSH INJURY

The surgical procedure is described in detail in Chapter III. The facial nerve was exposed near its exit from the SMF and followed proximally to reach its intratemporal segment. The dorsal and caudal aspects of the ear canal were incised to provide exposure of the tympanic bulla. The tympanic bulla was saucerized using a dental drill and the lateral bony aspect of the epitympanic region was removed. Ossicles were exposed and removed with jeweler's forceps to provide access to the region of the temporal bone located medially, where the intratemporal segment of the facial nerve lies. The bone covering the facial nerve was gradually thinned, beginning at the posterior aspect of the SMF and continuing antero-medially. Once sufficiently thinned within its course in the



facial canal, the facial nerve was crushed with a hemostat for 1 min. Gelfoam was lightly packed into the middle ear cavity prior to wound closure. In all animals, the left facial nerve was left intact to serve as an internal control.

For the intratemporal sham injury group, the drilling procedure was done as described above to expose the right facial nerve within the facial canal; but the nerve was left uncrushed. The post-operative survival time was 4 weeks for animals in the sham group. As the animals that received the intratemporal facial nerve crush showed delayed recovery, the post-operative survival time was extended to 8 weeks. Animals were sacrificed by CO<sub>2</sub> asphyxiation.

#### 4. FUNCTIONAL RECOVERY ASSESSMENT

Recovery of facial function on the right side was compared daily to the intact function on the left side, as described in Chapter III. Animals were observed for the return of eyeblink reflex and vibrissae orientation and vibrissae movement. The recovery time to reach a functional level equal to that on the uninjured side was noted for analysis. For the eyeblink reflex, in addition to its complete recovery, the time till onset of the blink reflex was also analyzed. Each group contained an *n* of 6-7.

#### 5. ELECTROMYOGRAPHIC RECORDINGS

Motor nerve conduction tests were done on anesthetized animals pre- and post-operatively on the day of axotomy and weekly thereafter (refer to Chapter III for details). Two stimulating electrodes were placed below the infraorbital ridge, two recording electrodes were placed on the vibrissae pad to record the evoked muscle activity distal to

stimulation, and a reference electrode was placed inferior to the ear canal (Fig. 16).

The facial nerve was stimulated at a frequency of 0.5 Hz with a 10 mA current, and an average of 25 responses was recorded for each trial. Peak amplitude was defined as the maximum evoked potential and latency as the time from the stimulation to the onset of response. Percent change in peak amplitude and latency, relative to the response on the unoperated side, was calculated and plotted as a function of weeks post-axotomy. An *n* of 4-9/time point was used for each of the 4 experimental groups.

## 7. FACIAL MOTONEURON SURVIVAL ASSESSMENT

Animals in all 4 experimental groups were sacrificed by CO<sub>2</sub> asphyxiation at 4 wpo, but the animals in the intratemporal crush were also sacrificed at an additional time point of 8 wpo. Brains were collected, and serial 40 µm cyrostat sections were taken fixed in 4% paraformaldehyde, and stained with thionin, as previously described in Chapter III. Slides were coded by one investigator and subsequently counted under blind conditions by another investigator. Cell profile counts were performed on every section, and the percentage change in surviving motoneurons between the left and right sides was calculated and compared between groups. Only surviving FMNs containing a clearly visible nucleus were counted (Serpe et al., 1999; Serpe et al., 2000; Serpe et al., 2003; Byram et al., 2003).

## 6. STATISTICAL ANALYSIS

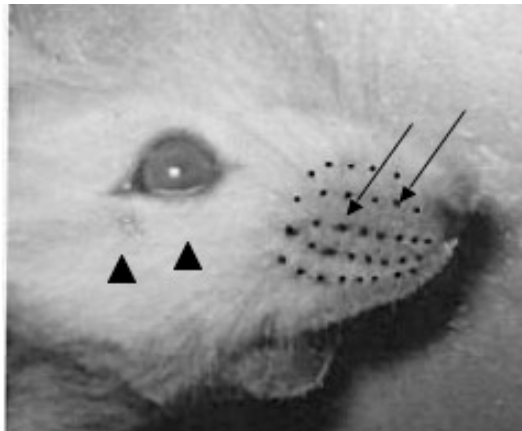
For each functional parameter, statistical significance of the differences in recovery times for the intratemporal and the extratemporal facial nerve injury models was

determined using unpaired t-tests. If any animals did not recover a functional parameter by the end of the study period (8 weeks), the recovery time was designated as >56 dpo. For some functional parameters, when all animals in a group did not recover function by 56 dpo, and thus an unpaired t-test could not be used, a one-sample t-test was done with the hypothetical value defined as 56. For motor nerve conduction studies, significant differences among the four experimental groups were determined using a two-way ANOVA (factors = days post-axotomy and treatment), followed by a Newman-Keuls' multiple comparison post-hoc test at  $p < 0.05$ . Statistical differences among cell survival in sham and injury groups were determined using a one-way ANOVA, followed by a Newman-Keuls' multiple comparison post-hoc test at  $p < 0.05$ .

#### D. RESULTS

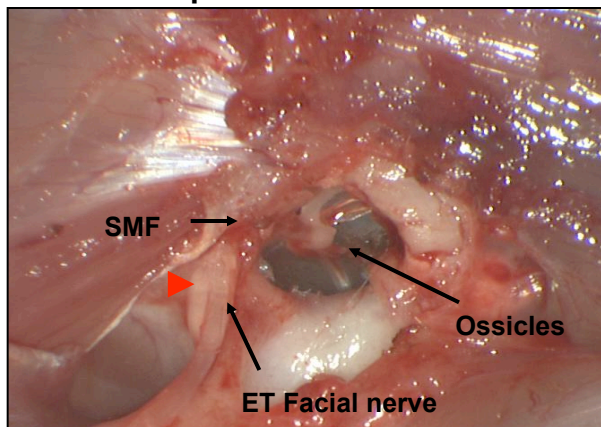
##### 1. DIFFERENCES IN FUNCTIONAL RECOVERY FOLLOWING EXTRATEMPORAL VS. INTRATEMPORAL FACIAL NERVE CRUSH

An intratemporal model of facial nerve injury was successfully developed. The distal, extratemporal site of facial nerve injury is compared to the proximal, intratemporal site in Figure 17. The intratemporal crush site was ~6-7 mm more proximal to the FMN than the extratemporal crush site. Figure 18 compares the return of facial nerve function in both models of facial nerve injury. The onset of the eyeblink reflex

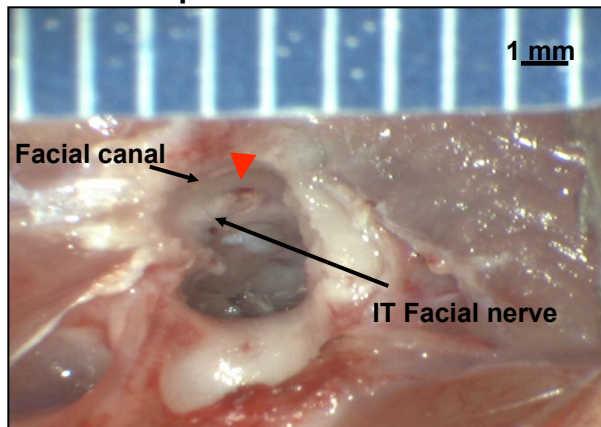


**Figure 16. Experimental setup for motor nerve conduction testing.** Diagram illustrates the placement of electrodes for measuring facial nerve conduction. Sites for the two recording electrodes on the vibrissal pad (arrows) and the two stimulating electrodes below the infraorbital ridge (arrowheads) are marked. The reference electrode was placed below the ear.

#### A. Extratemporal crush



#### B. Intratemporal crush

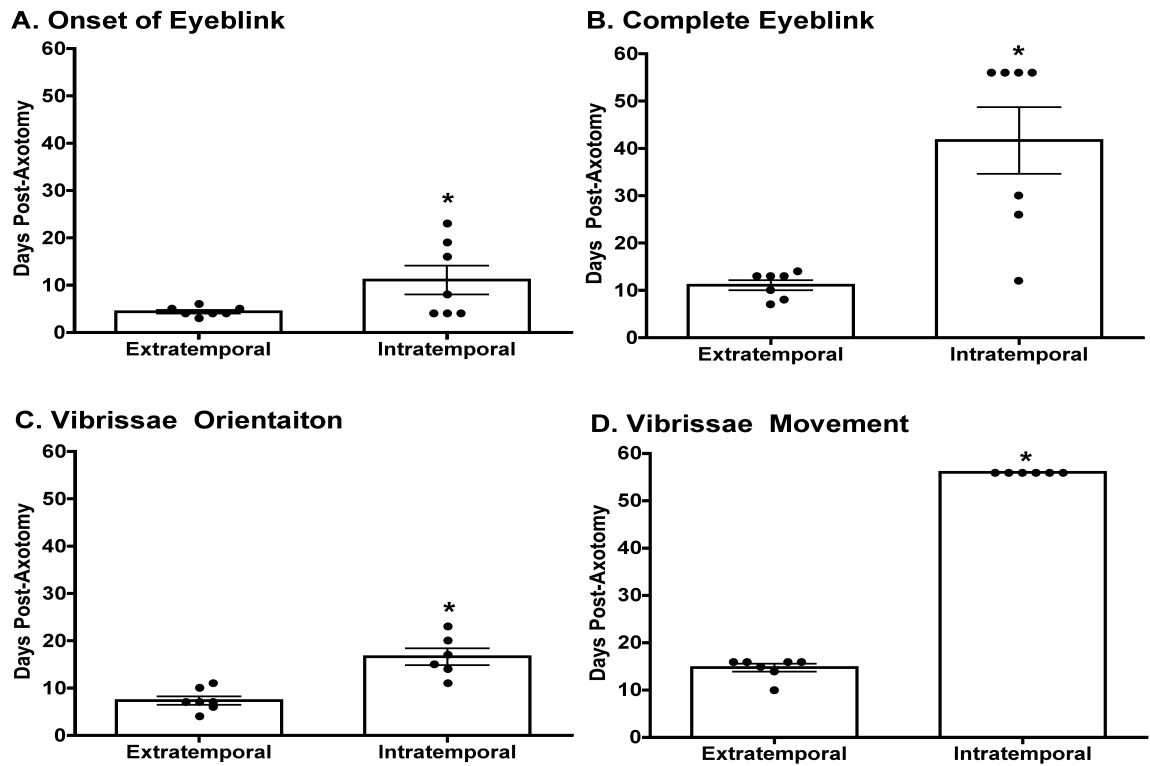


**Figure 17. Surgical design of extratemporal vs. intratemporal facial nerve injury.** Images illustrate extratemporal (A) and intratemporal (B) facial nerve segments. The red arrowheads represent the site of crush. A ruler with 1 mm marks is also presented in (B) as a scale. SMF: stylomastoid foramen; ET: extratemporal; IT: intratemporal.

represents initiation of functional recovery and was significantly delayed in animals receiving the intratemporal facial nerve crush as compared to the extratemporal crush (Fig. 18A). Following an extratemporal crush, the eyeblink first began to appear at  $4.43 \pm 0.37$  dpo, whereas an intratemporal crush led to a delayed onset at  $11.14 \pm 3.04$  dpo ( $p < 0.05$ ). The complete eyeblink reflex returned in animals receiving the extratemporal crush at  $11.14 \pm 1.06$  dpo (Fig. 18B). However, in animals receiving the intratemporal crush, return of the complete eyeblink was also significantly delayed to  $\geq 41.71 \pm 7.04$  dpo ( $p < 0.05$ ). Within this average recovery time for six animals, three animals recovered the full eyeblink reflex while the other three had an incomplete blink reflex even at the end of the study period of 56 dpo.

Normal vibrissae orientation returned in animals before vibrissae movement (Fig. 18C-D). While animals receiving the extratemporal crush displayed normal orientation by  $7.43 \pm 0.90$  dpo, animals with the intratemporal crush demonstrated significantly delayed return at  $16.67 \pm 0.76$  dpo (Fig. 18C;  $p < 0.05$ ). Unlike the eyeblink reflex, normal vibrissae orientation did return in all animals following an intratemporal crush. In the extratemporal facial nerve injury model, full vibrissae movement appeared at  $14.71 \pm 0.84$  dpo. (Fig. 18D) Comparatively, in all of the animals in the intratemporal injury group, vibrissae movement did not return to normal up till the end of the study period at 56 dpo ( $p < 0.05$ ).

As vibrissae movement is the last parameter to return, its return defines the recovery time for complete recovery of facial nerve function. Therefore, Figure 18D also shows that in animals receiving the extratemporal crush, facial function was complete at  $14.71 \pm 0.84$  dpo. An intratemporal injury of the facial nerve, however, resulted in



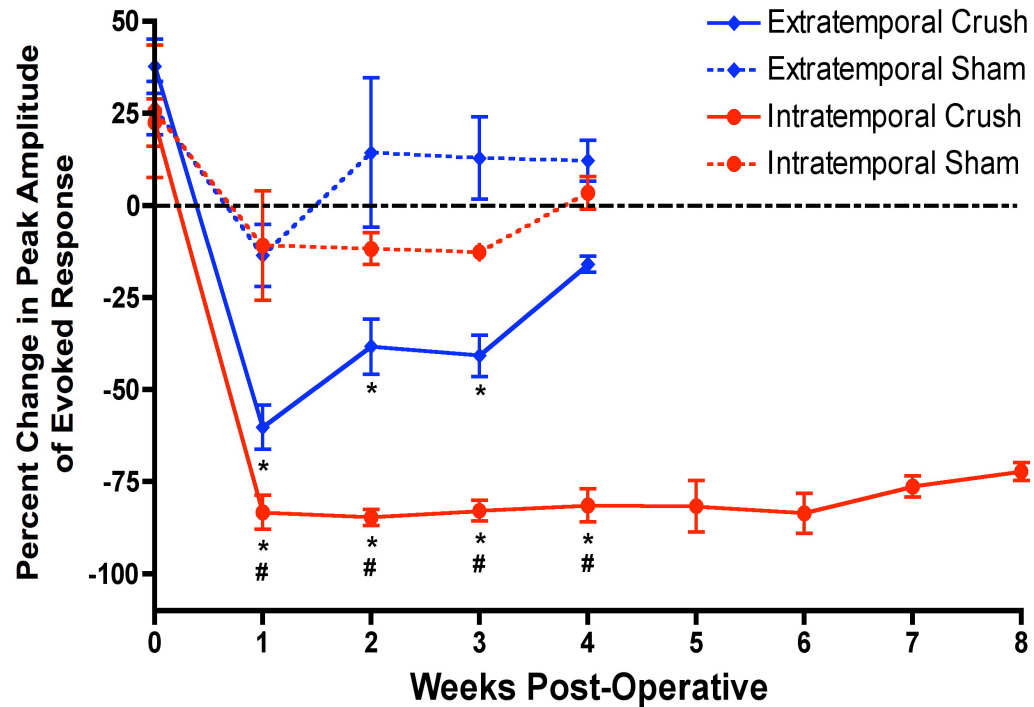
**Figure 18. Comparison of recovery of facial nerve functional parameters between extratemporal and intratemporal models of facial nerve crush injury.** Shown are recovery times for onset of the eyeblink reflex (A) and return of the full eyeblink reflex (B), vibrissae orientation (C), and vibrissae movement (D), following extratemporal and intratemporal facial nerve crush in rats. A scatter plot overlies each bar graph to display the spread of individual animals values within groups. Vertical lines represent standard error of the mean. *Asterisks* represent significant differences between groups ( $p < 0.05$ ).  $n = 6-7/\text{group}$ .

incomplete facial function at even 56 dpo ( $p<0.05$ ). Sham animals in the extratemporal and the intratemporal injury groups had an intact eyeblink reflex and vibrissae orientation and movement at 1 dpo, and thus they are not included in the graphs or analysis. Importantly, however, sham animals confirmed that the surgical procedure did not injure the facial nerve.

## 2. DIFFERENCES IN MOTOR NERVE CONDUCTION FOLLOWING EXTRATEMPORAL VS. INTRATEMPORAL FACIAL NERVE CRUSH

Motor nerve conduction studies were done weekly in the extratemporal and intratemporal sham and injury groups. Figure 19 demonstrates the percent change in the peak amplitude of evoked response for the crushed nerve, relative to the unoperated side. In both the extratemporal and intratemporal sham-operated animals, the percent change in peak amplitude remained around baseline, with no statistically decreased response post-injury as compared to the pre-injury time point at 0 wpo. Following an extratemporal injury, the peak amplitude significantly decreased to  $-60.16 \pm 6.00\%$  at 1 wpo, as compared to the  $37.73 \pm 7.33\%$  response prior to injury ( $p<0.05$ ). Relative to its sham group, the extratemporal injury group continued to have significantly lower peak amplitudes from 1-3 wpo (Fig. 19;  $p<0.05$ ). By 4 wpo following an extratemporal facial nerve injury, the peak amplitude increased to  $-15.95 \pm 2.24\%$ , a response not statistically different than that of the sham animals in this group ( $12.11 \pm 5.53\%$ ; Fig. 19).

Following an intratemporal facial nerve injury, the peak amplitude significantly dropped to  $-83.28 \pm 4.65\%$  at 1 wpo, as compared to the  $22.48 \pm 6.42\%$  response prior to injury ( $p<0.05$ ). With respect to its sham group, the intratemporal injury group had



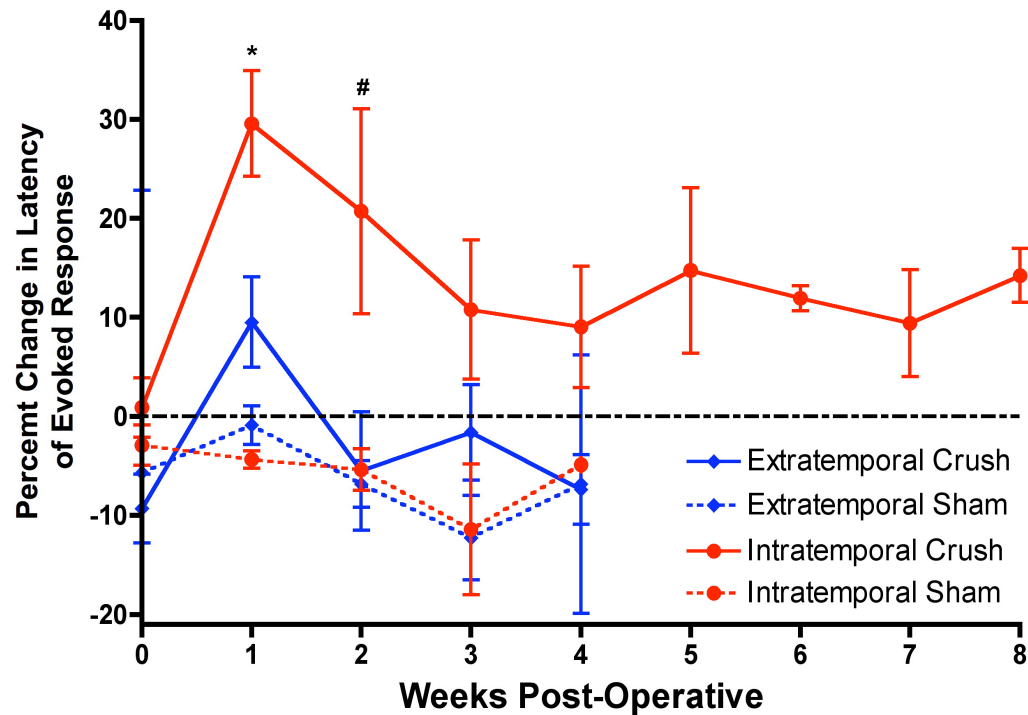
**Figure 19. Comparison of peak amplitude of evoked response between extratemporal and intratemporal models of facial nerve crush injury.** Motor nerve conduction testing was done to record peak amplitude of the evoked response in the vibrissal pad upon facial nerve stimulation. Shown are percent changes in peak amplitude on the injured side, relative to the unoperated side, over a time course. Vertical lines represent standard error of the mean. (\* =  $p < 0.05$  as compared to sham; # =  $p < 0.05$  as compared to extratemporal injury;  $n = 4-9$ /time point of each group). Dotted red line at y-intercept represents baseline.



significantly lower peak amplitudes from 1-4 wpo ( $p < 0.05$ ; Fig. 19). In addition, the peak amplitude failed to return to baseline levels even at 8 wpo, remaining at a value of  $-72.24 \pm 2.39\%$  ( $p < 0.05$  as compared to baseline). Also, in comparison to the extratemporal facial nerve injury, the intratemporal facial nerve injury led to significantly lower peak amplitudes from 1-4 wpo ( $p < 0.05$ ; Fig. 19). Therefore, while the amplitude of facial nerve conduction returned to normal in the extratemporal injury group, the intratemporal injury group displayed no improvement.

Figure 20 demonstrates the percent change in latency of response for the crushed facial nerve, relative to the unoperated side, over a course of 8 weeks following injury. In both the extratemporal and intratemporal sham-operated groups, the latency fluctuated around baseline pre- and post-injury. In animals receiving the extratemporal crush, the latency of response increased to  $9.53 \pm 4.56\%$ , a response not statistically different than that prior to injury ( $-9.29 \pm 3.46\%$ ). Furthermore, the percent change in latency did not significantly change following an extratemporal crush relative to its sham. At 1 wpo, latency increased to  $9.53 \pm 4.56\%$  in the extratemporal crush group, while the sham group had a latency change of  $-0.87 \pm 1.95\%$  ( $p > 0.05$ ; Fig. 20).

Following an intratemporal injury, however, the latency was significantly increased at 1 wpo relative to the sham group ( $29.58 \pm 5.35\%$  and  $-4.35 \pm 0.88\%$ , respectively; Fig. 20). By 2 wpo, the latency in the intratemporal injury group decreased to  $20.74 \pm 10.35\%$ , not statistically different than the  $-5.37 \pm 2.09\%$  change in the sham group. At 2 wpo, however, the latency of response in the intratemporal injury group was significantly increased compared to the extratemporal injury group ( $-5.50 \pm 5.97\%$ ;



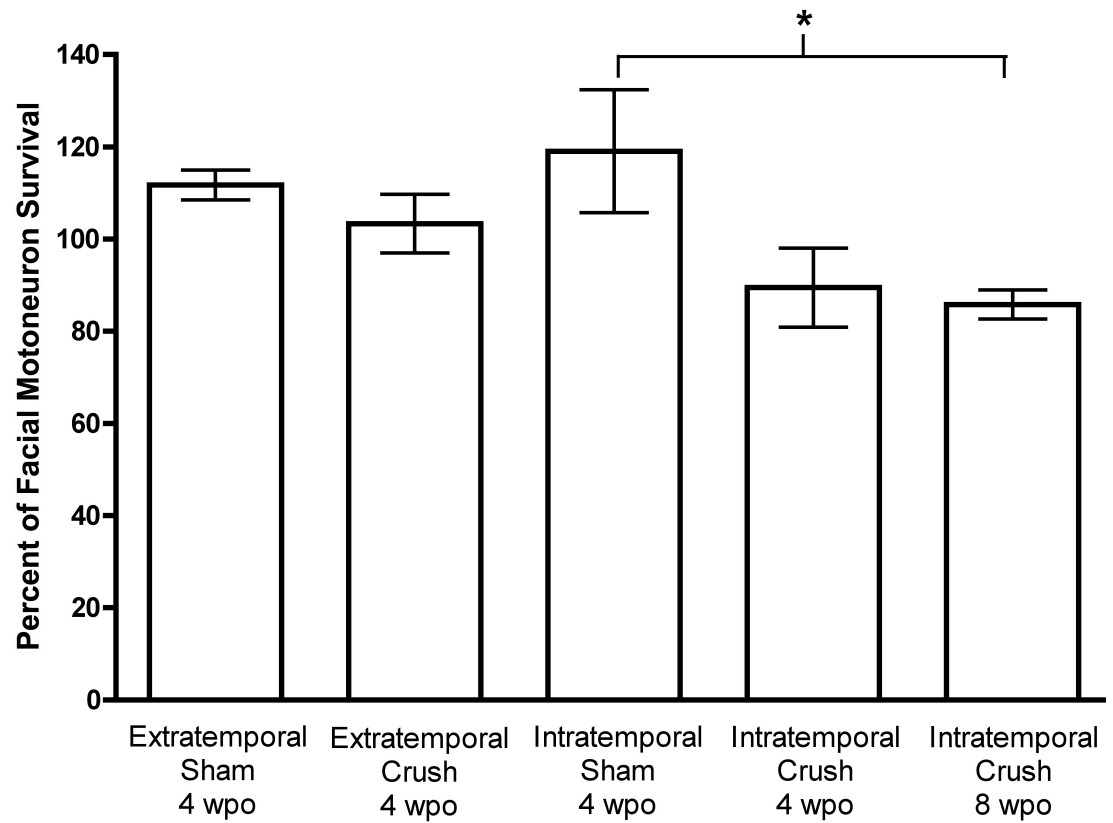
**Figure 20. Comparison of latency of evoked response between extratemporal and intratemporal models of facial nerve crush injury.** Motor nerve conduction testing was done to record latency of the evoked response in the vibrissal pad upon facial nerve stimulation. Shown are percent changes in latency on the injured side, relative to the unoperated side, over a time course. Vertical lines represent standard error of the mean. (\* =  $p < 0.05$  as compared to sham; # =  $p < 0.05$  as compared to extratemporal injury;  $n = 4-9$ /time point of each group). Dotted red line at y-intercept represents baseline.

$p < 0.05$ ; Fig. 20). Therefore, an intratemporal injury led to a longer latency in facial nerve conduction, comparative to not only sham but also extratemporal injury.

As animals receiving the extratemporal facial nerve injury recovered full function by ~2 wpo and reached peak amplitudes close to baseline levels by 4 wpo, this group was only followed until the end of 4 wpo. The intratemporal injury group was followed for a maximum of 8 wpo, in an attempt to capture return of functional recovery and normal motor nerve conduction. However, results demonstrate that complete facial function as well as evoked amplitude and latency of response to stimulation did not return to normal in animals receiving the intratemporal facial nerve injury even at the end of 8 wpo.

### 3. DIFFERENCES IN FACIAL MOTONEURON SURVIVAL FOLLOWING EXTRATEMPORAL VS. INTRATEMPORAL FACIAL NERVE CRUSH

Figure 21 demonstrates the percent of facial motoneurons survival in the two facial nerve injury models and their respective sham groups. At 4 wpo, the amount of cell survival in the animals receiving the extratemporal facial nerve crush was  $103.29 \pm 6.34\%$ , similar to the  $111.71 \pm 3.24\%$  survival in the extratemporal sham group (Fig. 21). Following an intratemporal facial nerve crush injury, cell survival in the FMN at 4 wpo was  $89.43 \pm 8.57$ , not significantly different than the  $119.03 \pm 13.35\%$  survival in the intratemporal sham group. As functional recovery in animals receiving the intratemporal facial nerve crush was incomplete at 4 wpo, cell counts were also assessed at 8 wpo. At 8 wpo, facial motoneurons survival in the intratemporal crush group dropped to  $85.78 \pm 3.15\%$ , significantly different than its respective sham group ( $p < 0.05$ ) but not different than the extratemporal crush group (Figure 21). Therefore, while a distal extratemporal



**Figure 21. Comparison of facial motoneuron survival between extratemporal and intratemporal models of facial nerve crush injury.** Percent survival of neurons within the right FMN was calculated relative to the left FMN. Motoneuron survival was assessed at 4 wpo for all groups and at an additional time point of 8 wpo in the intratemporal crush group. Vertical lines represent standard error of the mean. *Asterisk* represents significant difference between the intratemporal crush animals at 8 wpo and their respective sham.  $n = 3-5/\text{group}$ .

facial nerve crush did not induce any neuronal death, a proximal intratemporal crush lead to a ~15% loss of facial motoneurons.

## E. DISCUSSION

Facial nerve paralysis is a common peripheral nerve injury that has significant functional and emotional impact on patients. Due to the nerve's complex anatomical course, injuries can occur in different segments of the nerve (intracranial, intratemporal, and extratemporal), leading to different pathophysiological outcomes. Development of animal models representing the different types and locations of facial nerve injury is imperative for investigation of potential treatments. The current study compared the differences in functional recovery and motor nerve conduction in two models of facial nerve injury, the well-established extratemporal model and our newly developed intratemporal model. Results show that crushing of the facial nerve within its intratemporal segment leads to significantly delayed recovery in comparison to crushing of the nerve after its exit from the SMF. It is important to note that no animal model has previously characterized the recovery outcome following an intratemporal facial nerve injury. Therefore, the present findings have significant implications for patients with intratemporal facial nerve injuries as they may show hindered recovery and may require more assertive treatment strategies.

In the current study, all facial functional parameters and motor nerve conductance returned to normal following an extratemporal but not an intratemporal crush. Although the vibrissae orientation returned in animals receiving the intratemporal crush, the eyeblink reflex was incomplete in half of the animals at 56 dpo. Also at the end of 56

dpo, full vibrissae movement and thus complete functional recovery were incomplete in all of the animals receiving the intratemporal crush. In addition, motor nerve conduction study confirmed that the peak amplitude of the evoked response of the crushed facial nerve was still ~70% below that of the intact nerve at 56 dpo. As the amplitude of the evoked response is determined by the number of functioning motor nerve fibers, the current findings suggest that a majority of motor nerve fibers have not regenerated to the vibrissal musculature by 8 weeks following an intratemporal facial nerve injury (Oh and Shin, 2003). Latency, on the other hand, does return to normal by 2 weeks following an intratemporal injury. The latency of an evoked response is determined by how quickly the nerve impulse is conducted from the point of stimulation to the axonal terminal to result in muscle depolarization (Oh and Shin, 2003). As axonal fibers within a nerve bundle regenerate at different speeds, it is possible that a few fast-regenerating fibers may have reached the vibrissal musculature by 2 wpo to allow return of normal latency following an intratemporal crush. As latency is also affected by the myelination status of a nerve, recovery of any demyelinating changes may have occurred by 2 wpo.

Interestingly, although the severity of crush injury was similar in both extratemporal and intratemporal injury models, peak amplitude dropped to significantly lower levels following an intratemporal vs. an extratemporal injury at 1 wpo. It was expected that the initial fall in amplitude would be similar following injury at both location sites. A potential explanation for the higher amplitude in the extratemporal injury group is that since the first nerve conduction recording was taken at 1 wpo, the peak drop in amplitude may have occurred prior to that and thus may not have been

captured. It is important to note that the extratemporal and intratemporal sham-operated animals displayed no deficits in facial function and motor nerve conduction, verifying that the surgical procedure did not cause any additional damage to the facial nerve.

While it is unclear why recovery is significantly delayed following an intratemporal crush, there are several possible explanations. First, since the site of intratemporal injury is more proximal to the facial motor nucleus than the extratemporal injury, a greater neuronal cell loss was expected. A previous study by Mattson et al. (1999) found that transection of the facial nerve 0.5 mm from the brainstem led to ~75% neuronal loss whereas transection at the nerve's exit from the SMF led to ~25% loss. Although some studies have shown that peripheral nerve crush injuries do not lead to significant cell death, crush at sites more proximal to the cell body have not been reported (Swett et al., 1991; Swett et al., 1995). Our findings indicate that while an extratemporal crush led to 100% facial motoneuron survival, intratemporal crush led to a significantly greater neuronal loss of ~15% compared to its respective sham. A lack of statistical difference in cell survival between the extratemporal and intratemporal crush injuries may be due to large variability that needs to be reduced by increasing the  $n$  in each group.

A ~15% cell loss may not be expected to compromise return of facial nerve function to as great an extent as was observed in our model. However, cell loss may be more concentrated in some subpopulation of neurons within the FMN. The musculotopic organization of the rodent FMN is divided into 6 distinct subnuclei, innervating specific facial muscles (Komiya et al., 1984). The anterior auricular musculature is represented in the dorsal medial (DM) subnucleus, while the posterior auricular

musculature is represented in the ventral medial (VM) subnucleus. The mentalis and platysma muscles are represented mainly in the ventral intermediate (VI) and dorsal intermediate (DI) subnuclei, respectively. The orbicularis oculi and frontalis muscle are represented in the dorsal portions of the DI, DM, and dorsal lateral (DL) subnuclei. Finally, the nasolabial musculature is represented in both the DL and ventral lateral (VL) subnuclei. Our lab's previous studies in the mouse facial nerve axotomy model have found that following a transection of the nerve at its exit from the SMF, each subnucleus demonstrates a different pattern in the number of surviving neurons (Canh et al., 2006). While the VM and DM subnuclei are resistant to injury-induced cell loss, the VL subnucleus is more prone to degeneration. As the VL subnucleus sends axon projections to the vibrissae musculature, facial motoneuron loss may be concentrated to this region and thus may be one reason for incomplete functional recovery in animals receiving the intratemporal facial nerve crush. Interestingly, vibrissal movement is the functional recovery parameter that was always observed to be incomplete in animals receiving the intratemporal crush.

Another possible reason for the impeded functional recovery in the intratemporal injury model is misdirection of regenerating axons. Aberrant regeneration may occur when axons reach different muscle targets than their original targets during the recovery period. Although misdirection is more common in transection injuries, it has been reported to occur following crush injuries of multiply branched motor nerves (Hadlock et al., 2008). This phenomenon underlies the disabling condition of synkinesis often seen in patients with facial nerve injury. Following nerve injury in adult animals, the somatotopical organization of the facial motor nucleus is lost with respect to the



ophthalmic and buccal branches, contributing to misguided regeneration (Park et al., 1995). Synkinesis results in involuntary movement of one segment of the face while attempting to voluntarily move another part of the face. Currently, no therapy exists to prevent or treat synkinesis in patients who have suffered facial nerve injuries. Recently Hadlock et al. (2008) described development of an apparatus in rodent models that would allow assessment of eyeblink and whisking on both sides of the face to quantify synkinesis. Such a method could be used in the future to determine if synkinesis develops in our animal model of intratemporal facial nerve injury. If aberrant regeneration does prove to be the cause of hindered functional recovery in animals receiving an intratemporal facial nerve crush, then this injury model could further be used to test different therapies that may improve recovery from or prevent development of synkinesis.

Lastly, if the regeneration period is significantly prolonged, target muscles may undergo regressive alterations from the lack of reinnervation. Muscle atrophy of 20-90% has been reported in cases of long-standing facial nerve paralysis (Kumar et al., 2006). Additional changes characterize the progression of denervation-induced muscle atrophy, including degenerated myofibrils, fibrosis, capillary reduction and fatty infiltration. Therefore, atrophy of the facial musculature may be preventing return of functional recovery in the intratemporal injury model described in this study. Additional reasons for incomplete functional recovery may include a decreased ability of regenerating axons to traverse the crush zone or a lack of sustained neurotrophic support by neuronal and non-neuronal cells.

Facial nerve injuries have significant functional, emotional, and psychological impact patients. Current findings demonstrate that a more proximal injury of the facial nerve within its intratemporal segment leads to notably delayed recovery of function and motor nerve conductance as compared to the same injury performed more distally at the nerve's exit from the SMF. As a significant number of facial nerve injuries occur during the nerve's course within the temporal bone, the results have direct relevance for predicting the pathological and functional outcome in patients. Future studies may determine whether the delayed recovery seen with the intratemporal crush is due to an inability of the axons to traverse the injury site, misguided regeneration, or significant neuronal loss within certain subnuclei of FMN that is sufficient to compromise function. Furthermore, the intratemporal model of nerve injury described can now be used to investigate treatment strategies that may enhance regeneration and recovery.

**CHAPTER VII**  
**EFFECTS OF ELECTRICAL STIMULATION, TESTOSTERONE, AND**  
**PREDNISONE ON FUNCTIONAL RECOVERY FOLLOWING**  
**INTRATEMPORAL FACIAL NERVE INJURY**

**A. ABSTRACT**

Although various surgical techniques exist for repairing the facial nerve following injury, recovery is often suboptimal and treatments targeting the molecular mechanisms of regeneration are lacking. Results from Aim 1 and Aim 2 have demonstrated that administration of daily ES and systemic testosterone enhances regenerative properties and augments the growth program following an extratemporal crush of the facial nerve. The aim of the present study was to determine whether our combinatorial treatment strategy would be effective in the more clinically relevant intratemporal model of facial nerve injury, which demonstrates a substantially prolonged recovery time and induces significant facial motoneuron loss. Furthermore, ES and TP treatments were compared to prednisone treatment, a regimen currently used in the clinic for promoting recovery following injuries to the facial nerve within the temporal bone. A brief one-time ES, TP and prednisone treatment were administered in various combinations with each other following an intratemporal facial nerve crush injury in gonadectomized male rats. Results demonstrate that ES or TP treatment alone enhanced some functional parameters

more than the use of prednisone alone. Brief ES of the proximal nerve stump also accelerated the onset of functional recovery. None of the three treatments was effective in accelerating complete functional recovery or return of normal motor nerve conduction when administered alone. Animals given the combinatorial treatment of ES plus TP, however, demonstrated enhanced complete functional recovery and normal amplitude and latency of evoked response during motor nerve conduction testing. Therefore, our findings support that the combinatorial treatment strategy of using brief ES and TP together promises to be an effective therapeutic intervention even in the case of a more proximal injury of the facial nerve during its course in the temporal bone.

## B. INTRODUCTION

In 1821, Sir Charles Bell discovered that the facial nerve is responsible for facial muscle movement (Rosson and Redett, 2008). Understanding the anatomy of facial nerve has given way to development of several therapeutic strategies. Reconstructive treatments including direct anastomosis of the facial nerve stumps and nerve grafting procedures. Decompression of the facial canal is also used in cases of Bell's palsy with >90% degradation of facial nerve (Marais and Murray, 1995). In patients with chronic facial nerve damage, long-term pulsatile stimulation of facial musculature is recommended to promote reinnervation (Targan et al., 2000). Despite the advancements in technical repair of nerve injuries, recovery of function is frequently poor. The detection of herpes simplex virus in the endoneurial fluid of patients with Bell's palsy has led to the use of anti-viral agents in the past decade (May and Klein, 1991). Corticosteroid therapy is also widely used in cases of facial nerve inflammation and

edema. For both antiviral and steroid therapy, however, randomized controlled studies demonstrate conflicting results (Turk-Boru et al., 2005; Sullivan et al., 2007; Allen and Dunn, 2004; Salinas et al., 2004). As their efficacy remains controversial, further studies with larger patient populations and animal models need to be conducted.

As ES of injured nerves is associated with enhanced regeneration, it would be valuable to explore the translational potential of ES to facial nerve injury (Gordon et al., 2008). A summary of ES-mediated neuronal effects includes activating the excitability of neurons, upregulating expression of regeneration-associated genes, promoting regrowth of neurite after injury, improving selective targeting of regenerating axons, and enhancing the functional recovery of target organs (Al-Majed et al., 2000a; Brushart et al., 2005; Vivo et al., 2008; Gordon et al., 2003; Ahlborn et al., 2007; Zhang et al., 2007; Al-Majed et al., 2004; Al-Majed et al., 2000a). Brushart et al. (2002) have reported that ES improves axonal regeneration not by increasing the regeneration rate but by accelerating the growth of axons across the site of injury and suture repair in the rat femoral nerve model. Aim 1 of the present study also demonstrated that ES has no effect on accelerating the rate of regeneration of facial motor axons (Chapter IV). However, ES reduces the initial delay before sprout formation begins following crush of the extratemporal segment of the rat facial nerve. Results of Aim 2 further indicated that ES rapidly increases expression of regeneration-associated genes but the effects are transient (Chapter V). Based on these findings, a combinatorial approach would most likely be a valuable clinical tool, with an initiation technique such as ES followed by a treatment that targets rate of regeneration and maintains responsiveness at the end organs.

Use of gonadal steroids is one intervention that accelerates axonal regeneration rate. Following an extratemporal injury of the facial nerve, testosterone effectively increased the rate of facial nerve regeneration but failed to reduce the delay before sprout formation (Chapter IV). Aim 2 indicated that in comparison to the effects of ES, testosterone-mediated upregulation of regeneration-associated genes occurs later and is more sustained (Chapter V). In cases of regeneration over long distances, such as following an intratemporal facial nerve injury, administration of testosterone may especially be beneficial to ensure that a regenerative response is maintained over an extended time course.

The aim of the present study was to administer prednisone, ES, and testosterone alone and in combination with each other and to evaluate the effects of these treatments on functional recovery. Recovery of function following an intratemporal facial nerve injury was substantially delayed in comparison to extratemporal facial nerve injury (Chapter VI). An intratemporal facial nerve injury was also associated with a ~15% motoneuron loss. One reason for delayed recovery may be edema or an inflammatory response resulting from the injury within the facial canal that hinders regrowth of motor axons across the crush site. Therefore, this study examined the effects of a prednisone regime similar to the one used in humans with facial nerve injury, where a short-term, high dose prednisone was given orally and tapered off over a course of 10 days.

Several studies have confirmed that a one-time ES treatment, administered immediately following injury, is as effective as long-term stimulation in improving functional recovery, enhancing regeneration, and attenuating axotomy-induced cell loss (Gordon et al., 2007; Ahlborn et al., 2007; Okazaki et al. 2008; Al-Majed et al., 2000a).

Studies from our lab using the extemporal model of facial nerve injury have compared that the effects of various short-term periods of ES to daily stimulation (Foecking et al., unpublished data). Results demonstrate that just one, 30 min period of ES is no different than daily stimulation in improving recovery of facial nerve function. As short-term ES has more potential for clinical translation, the present study evaluates the effects of a one-time ES treatment. Results demonstrate significant differences in functional recovery among the various treatment groups, with prednisone having minimal effect and the combination of ES and testosterone enhancing complete functional recovery the most.

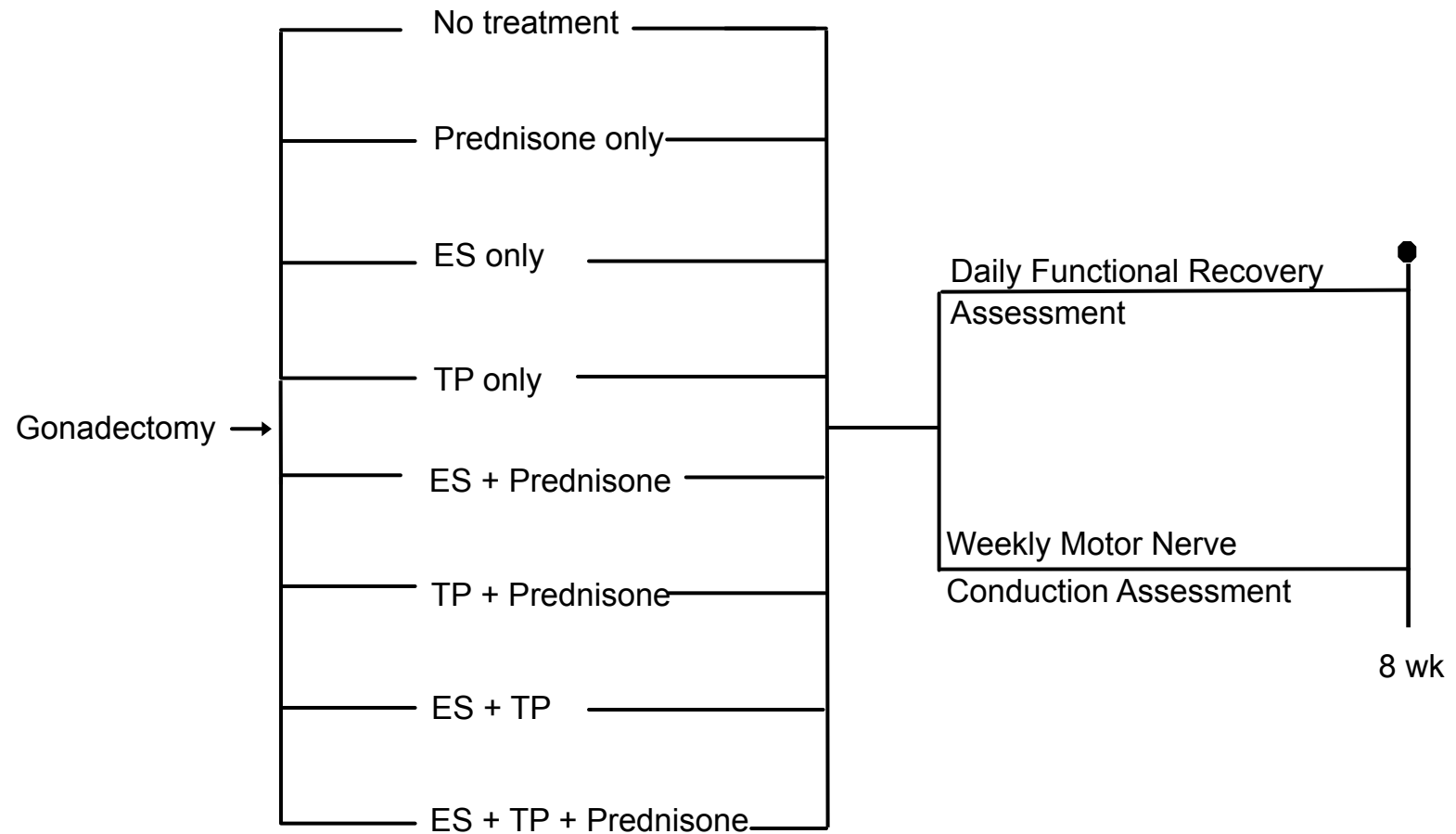
## C. MATERIALS AND METHODS

### 1. ANIMALS AND NERVE INJURY PARADIGM

Adult male Sprague-Dawley rats (~2 months old) were purchased from Harlan (Indianapolis, IN) and used for all experiments, as previously described in Chapter III. Three to five days prior to nerve injury, rats were anesthetized with isoflurane and castrated (refer to Chapter III for details). Figure 23 describes the experimental design for the present study. Following facial nerve injury, animals were divided into 8 experimental groups receiving various treatment combinations: [1] no treatment, [2] prednisone (P) only, [3] ES only, [4] TP only, [5] Stim + P, [6] TP + P, [7] Stim + TP, and [8] Stim + TP + P.

The surgical procedure for the intratemporal facial nerve injury was performed as described in Chapter III. The facial nerve was exposed near its exit from the SMF and

*Intratemporal Facial Nerve Crush Plus:*



**Figure 22: Experimental Design for Aim 4.** This timeline will be followed for all 8 experimental groups.



followed proximally to reach its intratemporal segment. The tympanic bulla and the epitympanic region were exposed and drilled in a sacuerized fashion. Ossicles were exposed and removed with jeweler's forceps to provide access to the region of the temporal bone located medially, where the intratemporal segment of the facial nerve lies. The bone covering the facial nerve was gradually thinned, beginning at the posterior aspect of the SMF and continuing antero-medially. Once sufficiently thinned within its course in the facial canal, the facial nerve was crushed with fine jeweler's forceps for 1 min. Gelfoam was lightly packed into the middle ear cavity prior to wound closure. In all animals, the left facial nerve was left intact to serve as an internal control.

## 2. ELECTRICAL STIMULATION

Two Teflon-coated wires, bared of insulation for 2-3mm, were soldered to two "male" connector pins in a connector strip (refer to Chapter III for details). Anesthetized animals received a one-time ES treatment during the axotomy surgery. Prior to administration of an intratemporal facial nerve crush injury, electrodes were sutured in place as described previously, with one electrode adjacent to the nerve and another 3-5 mm away. The facial nerve was briefly stimulated for <5 sec before axotomy so that the voltage threshold for evoking a facial nerve response could be determined. The voltage at which vibrissae twitching were elicited was used as the stimulating voltage. Immediately after axotomy, the facial nerve was stimulated proximal to the crush injury site at a frequency of 20 Hz for 30 min. After the stimulation period, the electrodes were removed and wound sites were closed as described above.

### 3. STEROID ADMINISTRATION

For administration of TP, a small incision was made on the mid-dorsal surface of animals. A subcutaneous pocket was created for implantation of two Silastic capsules (0.062 in. id x 0.095 in. od; 10-mm length), equilibrated in physiological saline and containing 100% crystalline TP. Capsules were implanted immediately following axotomy to establish supraphysiological levels of systemic TP (Kujawa et al., 1989; Hetzler et al., 2008; Tanzer and Jones, 2004).

A tapering dose of prednisone (dissolved in physiological saline) was administered orally using a 2", 18-gauge gavage needle. Starting on immediately following axotomy, a dose of 2.5 mg/kg was administered over a course of 11 days at the following intervals: twice daily for the first three days, once daily for the next 3 days, once every other day for the subsequent 3 days. Animals not receiving the prednisone treatment received similar volumes of vehicle (saline) by oral gavage at the same dosing intervals.

### 4. FUNCTIONAL RECOVERY ASSESSMENT

Recovery of facial function on the right side was compared daily to the intact function on the left side, as described previously in Chapter III. Animals were observed for the return of eyeblink reflex and vibrissae orientation and vibrissae movement. For each of these functional parameters, the recovery time to reach function symmetric to that on the uninjured side was noted for analysis. For the eyeblink reflex, in addition to its complete recovery, the time till onset of the blink was also analyzed. Each group contained an *n* of 5-6.

## 5. ELECTROMYOGRAPHIC RECORDINGS

Motor nerve conduction tests were done as described previously in Chapter III. Animals were anesthetized with intra-peritoneal injections Ketamine-Xylazine, and recordings were taken pre- and post-operatively on the day of axotomy and weekly thereafter. Two stimulating electrodes were placed below the infraorbital ridge, and two recording electrodes were placed on the vibrissae pad to record the evoked muscle activity distal to stimulation. A reference electrode was placed inferior to the external ear. The facial nerve was stimulated at a frequency of 0.5 Hz and a 15 mA current, and an average of 25 responses was recorded. Percent change in peak amplitude and latency, relative to the response on the unoperated side, was calculated and plotted as a function of weeks post-axotomy. An *n* of 4-6/time point was used for each of the 8 experimental groups.

## 6. STATISTICAL ANALYSIS

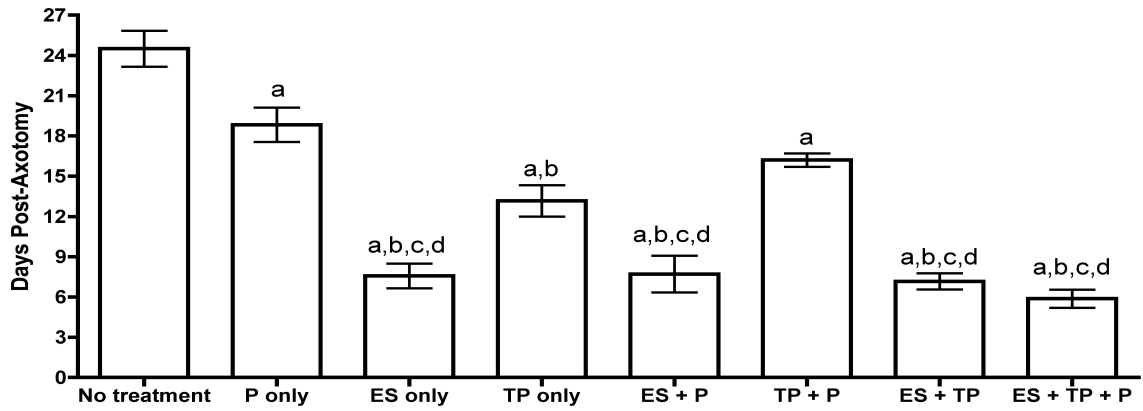
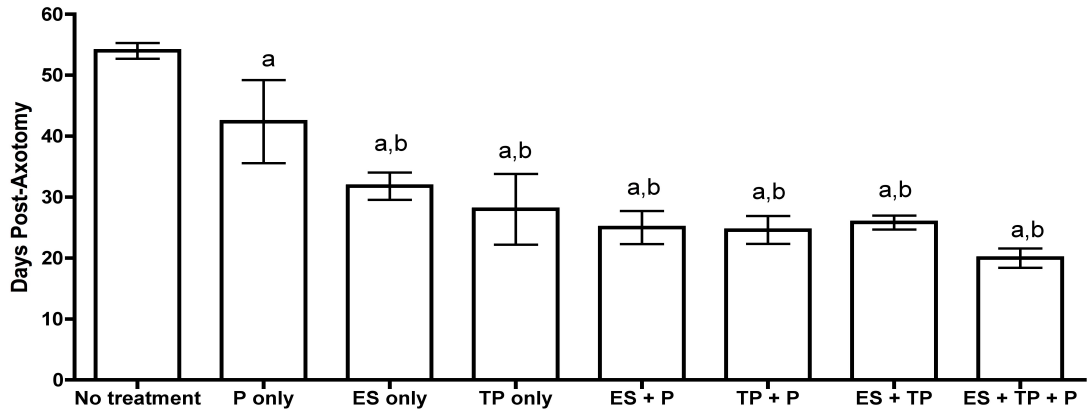
Significant differences in functional recovery parameters among the various treatment groups were assessed using a one-way ANOVA, followed by a Newman-Keul's post-hoc test at  $p < 0.05$ . To determine statistical significance among groups for percent change in amplitude and latency, a two-way ANOVA (factors = dpo and treatment), followed by a Newman-Keuls's post-hoc test at  $p < 0.05$ , was performed. For ease of visual comparison, groups are often presented separately on graphs.

## D. RESULTS

### 1. DIFFERENCES IN FUNCTIONAL RECOVERY FOLLOWING TREATMENT WITH PREDNISONE, ES, AND/OR TP

Figure 23 demonstrates the effects of ES, TP, and/or prednisone on the timing of the onset and full recovery of the eyeblink reflex. Administration of prednisone significantly accelerated the onset of the eyeblink reflex as compared to no treatment ( $18.8 \pm 1.3$  dpo and  $24.5 \pm 1.3$  dpo, respectively;  $p < 0.05$ ; Figure 23A). Administration of a brief ES treatment accelerated the onset of the eyeblink reflex to  $7.6 \pm 0.6$  dpo, a significantly shorter recovery time than both the no treatment and the prednisone-only groups ( $p < 0.05$ ). TP administration by itself significantly shortened the time for the onset of the eyeblink reflex ( $11.7 \pm 1.4$  dpo), as compared to no treatment and prednisone alone ( $p < 0.05$ ). Adding prednisone treatment to ES or TP had no additional effect, as compared to ES or TP alone. With ES plus prednisone, the onset of the eyeblink reflex occurred at  $7.7 \pm 1.4$  dpo and with TP plus prednisone, the onset of the eyeblink reflex occurred at  $16.2 \pm 0.5$  dpo ( $p < 0.05$  relative to no treatment). Administration of ES plus TP or administration of ES, TP, plus prednisone had similar effects in accelerating the onset of the eyeblink reflex ( $7.2 \pm 0.6$  dpo and  $5.9 \pm 0.8$  dpo, respectively), as compared to no treatment ( $p < 0.05$ ). Importantly, administration of ES, alone or in combination with other treatments, shortened the time to onset of the eyeblink reflex even more than TP and/or prednisone treatments ( $p < 0.05$ ).

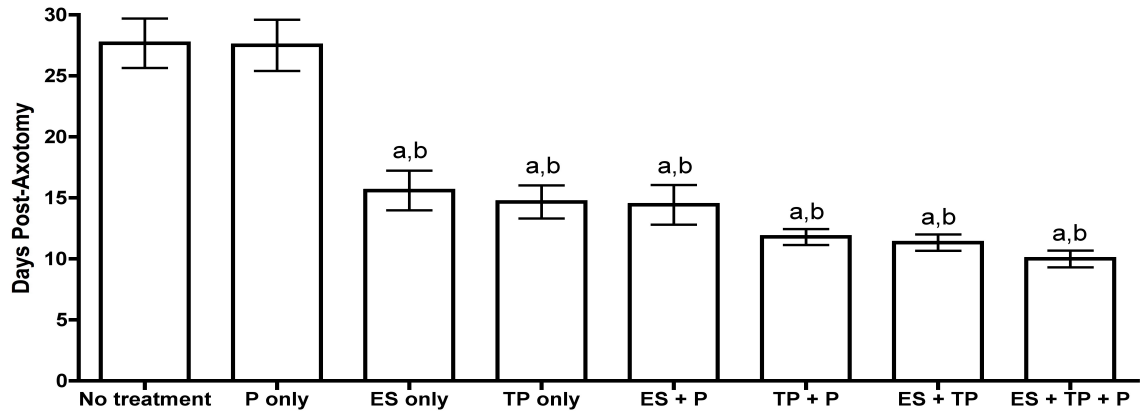
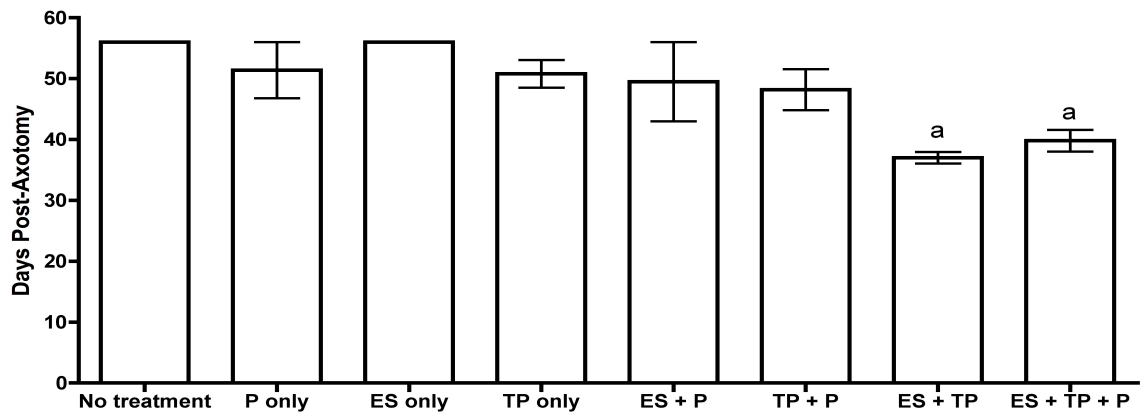
Recovery time of the full eyeblink reflex was significantly shortened in all treatment groups, relative to  $54 \pm 1.3$  dpo in the no treatment group (Figure 23B). Within

**A. Onset of Eyeblink Reflex****B. Complete Eyeblink Reflex**

**Figure 23. Effects of prednisone, brief ES, and/or TP on onset and complete return of the eyeblink reflex following an intratemporal facial nerve crush injury.** Shown are recovery times for the onset of the eyeblink reflex (A) and return of the complete eyeblink reflex (B) following administration of various combinations of prednisone (P), ES, and TP treatments. As animals were followed for a maximum of 56 dpo, incomplete return of any functional parameter was designated a recovery time of 56 dpo. Vertical lines represent standard error of the mean. (a =  $p < 0.05$ , relative to no treatment; b =  $p < 0.05$ , relative to prednisone only; c =  $p < 0.05$ , relative to TP only; d =  $p < 0.05$ , relative to TP + P).  $n = 5-8/\text{group}$ .

the untreated group, only one-third of the animals recovered the full eyeblink reflex by the maximum post-operative survival time of 56 dpo. The remaining two-thirds had an incomplete eyeblink even at 56 dpo. Relative to no treatment, administration of prednisone significantly shortened the recovery time for the complete eyeblink reflex to  $33.3 \pm 7.2$  dpo. ES and TP treatments alone also significantly accelerated the recovery of the full eyeblink to a similar extent ( $31.8 \pm 2.3$  dpo and  $28 \pm 5.3$  dpo, respectively;  $p < 0.05$  relative to no treatment). Addition of prednisone to ES or TP had no supplemental effect in enhancing recovery time for the full eyeblink, as recovery occurred at  $25 \pm 2.7$  dpo with ES plus prednisone and at  $24.6 \pm 2.3$  dpo with TP plus prednisone. Administration of ES plus TP or administration of ES, TP, plus prednisone had similar effects in accelerating the onset of the eyeblink reflex ( $25.8 \pm 1.1$  dpo and  $20 \pm 1.6$  dpo, respectively), relative to no treatment ( $p < 0.05$ ). In conclusion, recovery of the complete eyeblink reflex was enhanced in the presence of any treatment; however, the effects were more pronounced with the administration of ES and/or TP as compared to prednisone.

Vibrissae orientation returned in untreated animals at  $27.7 \pm 2.0$  dpo (Figure 24B). Prednisone administration had no effect on the recovery time for return of vibrissae orientation ( $27.5 \pm 2.1$  dpo). Relative to the no treatment and the prednisone-only groups, treatment with ES significantly accelerated recovery of vibrissae orientation to  $15.6 \pm 1.6$  dpo ( $p < 0.05$ ). Administration of TP also significantly shortened the recovery time for vibrissae orientation to  $14.7 \pm 1.4$  dpo, relative to no treatment and prednisone treatment ( $p < 0.05$ ). Addition of prednisone to ES or TP had no supplemental effect in enhancing recovery time for return of vibrissae orientation, as recovery occurred

**A. Complete Vibrissae Orientation****B. Complete Vibrissae Movement**

**Figure 24. Effects of prednisone, brief ES, and/or TP on return of vibrissae orientation and movement following an intratemporal facial nerve crush injury.** Shown are recovery times for return of normal vibrissae orientation (A) and full vibrissae movement (B) following administration of various combinations of prednisone (P), ES, and TP treatments. As animals were followed for a maximum of 56 dpo, incomplete return of any functional parameter was designated a recovery time of 56 dpo. Vertical lines represent standard error of the mean. (a =  $p < 0.05$ , relative to no treatment; b =  $p < 0.05$ , relative to prednisone only).  $n = 5-8/\text{group}$ .

at  $16.8 \pm 1.8$  dpo with ES plus prednisone and at  $11.8 \pm 0.8$  dpo with TP plus prednisone. Administration of ES plus TP or administration of ES, TP, and prednisone had similar effects in accelerating the return of vibrissae orientation ( $11.3 \pm 0.7$  dpo and  $10 \pm 0.7$  dpo, respectively), as compared to no treatment ( $p < 0.05$ ). Therefore, ES- or TP-treated animals displayed accelerated recovery of vibrissae orientation relative to prednisone-treated and untreated animals.

As vibrissae movement is the last facial functional parameter to return, the recovery time for vibrissae movement also determines the time for complete recovery. In all animals receiving no treatment, the recovery treatment for vibrissae movement was  $>56$  dpo (Figure 24B). Prednisone treatment did not significantly alter vibrissae movement recovery time ( $\geq 51.4 \pm 4.6$  dpo), relative to no treatment. ES or TP treatments alone, or each in combination with prednisone, were also ineffective in shortening recovery of complete vibrissae movement (ES only =  $>56$  dpo; TP only =  $50.8 \pm 2.3$ ; ES + prednisone =  $\geq 49.5 \pm 6.5$  dpo; TP + prednisone =  $\geq 48.2 \pm 3.4$  dpo). Administration of the ES plus TP combinatorial treatment significantly reduced the recovery time for complete vibrissae movement to  $37 \pm 1.0$  dpo, relative to no treatment ( $p < 0.05$ ). Animals receiving ES, TP plus prednisone also displayed a similar enhancement in return of vibrissae movement, with a recovery time of  $39.8 \pm 1.8$  dpo ( $p < 0.05$  relative to no treatment). Therefore, only administration of ES and TP together was effective in accelerating recovery of vibrissae movement.

Table 3 breaks down the number of animals within each treatment group that did or did not attain complete facial functional recovery. All of the animals in the untreated group displayed incomplete facial function at 56 dpo. In the prednisone treatment group,



**Table 3. Percentage of animals achieving complete facial functional recovery following an intratemporal facial nerve crush**

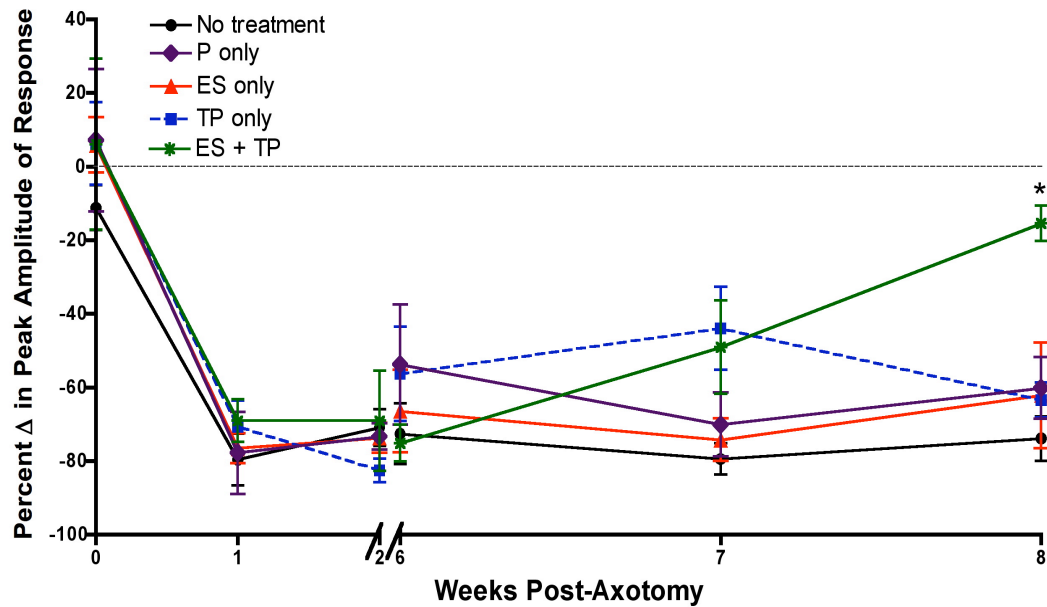
<b>Treatment Group</b>	<b>Animals with Complete Facial Functional Recovery</b>	<b>Animals with Incomplete Facial Functional Recovery</b>
No treatment	0%	100%
P only	20%	80%
ES only	0%	100%
TP only	60%	40%
ES + P	20%	80%
TP + P	60%	40%
ES + TP	100%	0%
ES + TP + P	100%	0%

20% of the animals attained complete facial function while the remaining 80% still displayed incomplete recovery at 56 dpo. While 100% of the animals receiving ES treatment did not achieve complete recovery, 60% of the animals receiving TP treatment did regain full facial function. When prednisone was added to ES or TP, 20% of the animals in the ES plus prednisone group and 60% in the TP plus prednisone group achieved complete recovery of facial nerve function. Lastly, in both the ES plus TP and the ES plus TP plus prednisone groups, 100% of the animals displayed complete regain of facial nerve function.

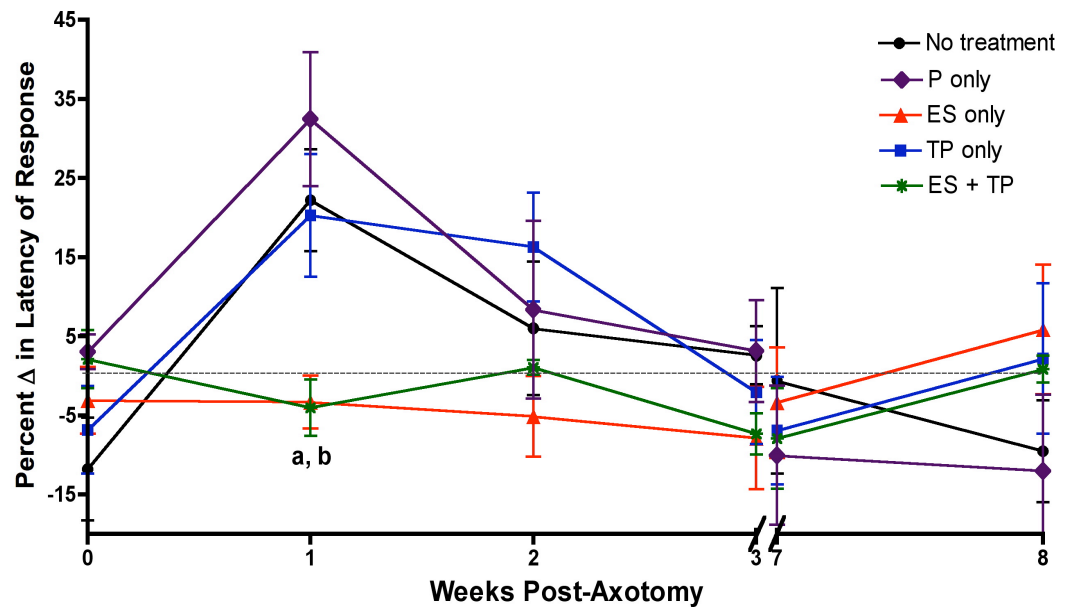
## 2. DIFFERENCES IN MOTOR NERVE CONDUCTION FOLLOWING TREATMENT WITH PREDNISONE, ES, AND/OR TP

Motor nerve conduction studies were performed weekly following axotomy and treatment with prednisone, ES, and/or TP. Figure 25 demonstrates the percent change in peak amplitude of the evoked response for the crushed facial nerve in animals receiving no treatment, individual treatments (prednisone, ES, or TP), and the combination of ES plus TP. Following axotomy at 1 wpo, peak amplitude dropped to -70-80% in all groups. By 8 wpo, peak amplitude in untreated animals or animals receiving individual treatments (prednisone, ES, or TP) remained ~60-70% below normal. In comparison, in animals receiving the combined treatment of ES plus TP, the peak amplitude levels returned to normal baseline values ( $-15.4 \pm 4.8\%$ ) at 8 wpo ( $p < 0.05$ ).

Figure 26 demonstrates the percent change in latency of the evoked response for the crushed facial nerve in animals receiving no treatment, individual treatments (prednisone, ES, or TP), or the combination of ES plus TP. Following axotomy at 1 wpo,



**Figure 25. Effects of prednisone, brief ES, and/or TP on peak amplitude of evoked response following an intratemporal facial nerve crush injury.** Motor nerve conduction testing was done to record peak amplitude of the evoked response in the vibrissal pad upon facial nerve stimulation. Shown are percent changes in peak amplitude on the injured side, relative to the unoperated side, over a time course. Vertical lines represent standard error of the mean. (\* =  $p < 0.05$  as compared to no treatment;  $n = 4-7$ /time point of each group). Dotted red line at y-intercept represents baseline. (P = prednisone).



**Figure 26. Effects of prednisone, brief ES, and/or TP on latency of evoked response following an intratemporal facial nerve crush injury.** Motor nerve conduction testing was done to record latency of the evoked response in the vibrissal pad upon facial nerve stimulation. Shown are percent changes in latency on the injured side, relative to the unoperated side, over a time course. Vertical lines represent standard error of the mean. (a =  $p < 0.05$ , relative to no treatment; b =  $p < 0.05$ , relative to prednisone only;  $n = 4-7$ /time point of each group). Dotted line at y-intercept represents baseline. (P = prednisone).

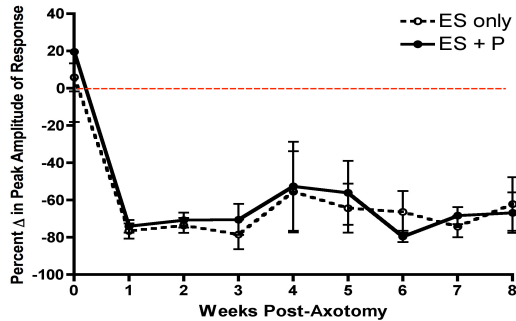
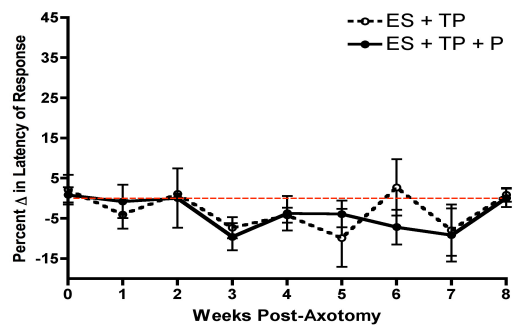
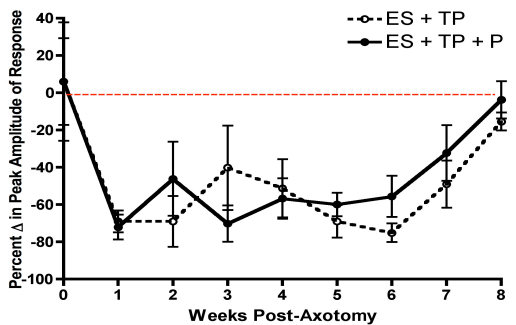
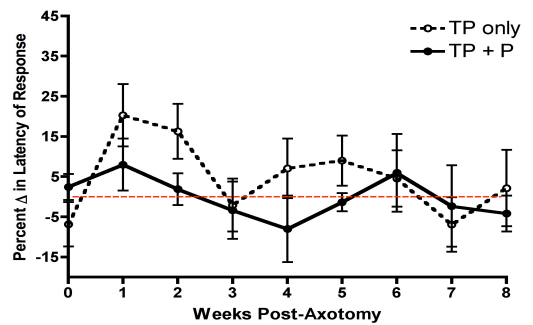
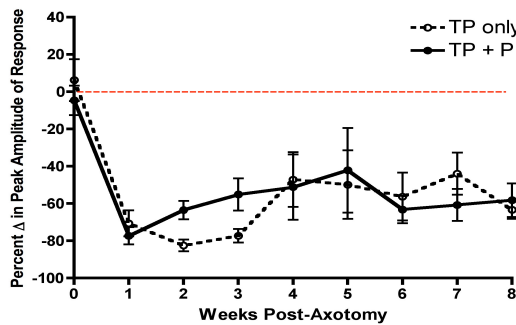
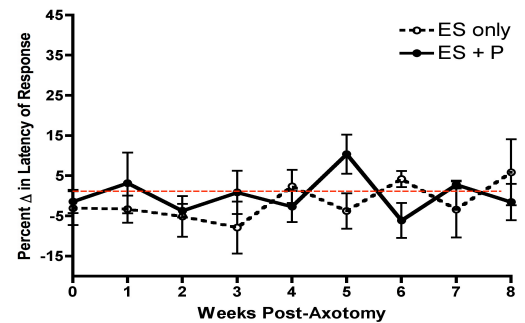
latency of response increased by ~20-30% in animals receiving no treatment, prednisone alone, or TP alone. However, in animals receiving ES, no increase in latency was noted. Rather the latency remained around normal baseline values in the ES only and the ES plus TP groups ( $-3.3 \pm 3.4\%$  and  $-4.0 \pm 3.5\%$ , respectively;  $p < 0.05$  relative to no treatment).

Figure 27 compares the percent change in peak amplitude and latency of the evoked response for the ES, TP and ES plus TP treatments with or without the addition of prednisone. Administration of prednisone had no additional effects as compared to the ES, TP, or ES plus TP treatments alone. The amplitude and latency remained below normal at 8 wpo unless both ES and TP were administered together.

## E. DISCUSSION

The present study found that administration of prednisone, ES, and testosterone had varying effects on functional recovery. Although prednisone alone improved recovery of some functional parameters (eyeblink reflex), it did not significantly affect functional recovery. ES and testosterone alone accelerated most functional recovery parameters to a greater extent than prednisone, but were still insufficient to improve complete functional recovery as compared to untreated animals. The combination of ES and testosterone, however, significantly shortened the time till complete functional recovery, demonstrating the benefit of a combinatorial treatment approach.

Although corticosteroids are widely used in idiopathic facial nerve paralysis, their efficacy has not been clearly demonstrated. In a randomized, prospective controlled study, patients were given steroids within the first 3 days after the onset of symptoms

**A. Amplitude of Evoked Response****B. Latency of Evoked Response**

**Figure 27. Effects of the addition of prednisone to ES only, TP only, and ES plus TP treatments on motor nerve conduction following an intratemporal facial nerve crush injury.** Motor nerve conduction testing was done to record peak amplitude and latency of the evoked response in the vibrissal pad upon facial nerve stimulation. Shown are percent changes in amplitude (A) and latency (B) on the injured side, relative to the unoperated side, over a time course. Vertical lines represent standard error of the mean. Dotted line at y-intercept represents baseline.  $n = 3-7$ /time point of each group. (P = prednisone).

(Turk-Boru et al., 2005). Although the initial response seemed to improve symptoms, by the end of 6 weeks, steroids had no significant effects as compared to no treatment. In a retrospective study evaluating the effects of corticosteroid therapy, results demonstrated a non-significant difference, with a similar percentage of treated and untreated patients having incomplete recovery and cosmetically disabling sequelae six months after the onset of facial nerve paralysis (Salinas et al., 2004). Prednisone has also been shown to be ineffective in promoting early recovery from post-parotidectomy facial nerve paralysis in a prospective, randomized trial (Roh and Park, 2008). Although some studies do support the idea that corticosteroids shorten the time to complete recovery (Engstrom et al, 2008), evidence is inconclusive. A similar short-term, high dose methylprednisolone treatment following optic nerve injury in rats was found to have no effect on cell survival, macrophage activity at the site of injury, axonal regeneration, or visual function (Ohlsson et al., 2004). Following spinal cord injury in dogs, administration of methylprednisolone with DC electrical field improved function more than the use of either treatment alone (Shen et al., 2005). In the current study, prednisone promoted earlier recovery of the eyeblink reflex but had no significant effect on complete recovery or return of normal nerve conduction. When combined with ES or TP, prednisone had no additional effect. Therefore, either the dose of prednisone administered may have been insufficient to induce recovery, or prednisone as a treatment strategy may be ineffective in enhancing regeneration.

Following nerve transection and surgical repair, investigators have described axonal regeneration to be a “staggered” process, in which axons cross the injury site at asynchronous rates (Brushart et al., 2002; Al-Majed et al., 2000a). Furthermore, axons

may branch repeatedly to enter several different endoneurial tubes, and some evidence also exists for axons proceeding backwards in search for distal nerve stumps (Witzel et al., 2005). Although such events may be more commonly seen following transection and surgical repair of nerves rather than crush injuries, regeneration of axons across the injury site may nevertheless be a rate-limiting process. As 1 h ES has been shown to substantially reduce the time for femoral motoneurons to regenerate axons across a suture site, the brief ES treatment in our intratemporal facial nerve injury model may also be accelerating growth across the crush site.

Compared to no treatment and prednisone or testosterone treatments, ES shortened the time until the onset of the eyeblink reflex, suggesting that it helps initiate regeneration earlier. Results from motor nerve conduction studies further demonstrated that administration of ES prevented the increase in latency of evoked response at 1 wpo. As the fastest arriving fibers of a nerve determine latency, ES may either have induced some of the fastest-growing axons to regenerate far enough to conduct the nerve impulse at a normal rate or it may have attenuated any demyelination associated with injury. However, ES administered by itself does not promote return of normal amplitude of the evoked facial nerve response. Also, none of the animals receiving ES alone were able to reach complete functional recovery at the end of the study period. Therefore, although ES may be effective in starting up the regeneration response, it may fail to promote recovery in the long run.

In the extratemporal model of facial nerve injury, our studies have demonstrated that androgens increase the overall regeneration rate. Testosterone administration alone in the present study did accelerate recovery of the eyeblink reflex and vibrissae



orientation, as well as complete recovery in 60% of the animals, a percentage greater than that achieved by prednisone or ES treatments. In addition to its effects on axonal regenerative properties, testosterone may also be affecting the target musculature. In untreated animals, prolonged denervation may be inducing muscle atrophy that would further prevent regain of complete facial function. Testosterone has been demonstrated to decrease expression of the ubiquitin ligase muscle atrophy factor, termed MAFbx, which is associated with denervation-induced muscle loss (Zhao et al., 2008). Testosterone represses the MAFbx promoter via an AR-dependent mechanism. AR receptor is found in muscles that are highly responsive to androgens and AR expression is enriched at the neuromuscular junctions. Therefore, the facial muscles may also be sites of androgen action that aid in prevention of muscle atrophy and/or promotion of reinnervation. In the adult rat sciatic nerve, AR mRNA levels are also localized in the endoneurial compartment, specifically in the endoneurial fibroblasts and endothelial cells, and within Schwann cells (Jordan et al., 2002). These additional sites of androgen action may also maintain a regenerative-permissive environment over a prolonged period.

The effectiveness of the combinatorial treatment of ES and testosterone in substantially accelerating functional recovery and return of normal motor nerve conduction may be explained by faster regrowth across the crush site, stimulated by ES, in addition to an enhanced regenerative response sustained by TP. These findings have direct clinical relevance for cases of intratemporal facial nerve injury in which functional recovery is significantly prolonged. Furthermore, results imply that ES and testosterone may serve as better treatment strategies than the use of corticosteroids in facial nerve injury. Corticosteroid treatment is contraindicated in children and patients with

conditions such as diabetes, liver disease, immunodeficiency, and glaucoma. As

Bell's palsy is 4 times more likely to occur in diabetics and in people over the age of 65, such patient populations may benefit from other treatment strategies (Finsterer, 2008).

Therefore, the combinatorial approach using ES and testosterone may be a valuable clinical tool.

## **CHAPTER VIII**

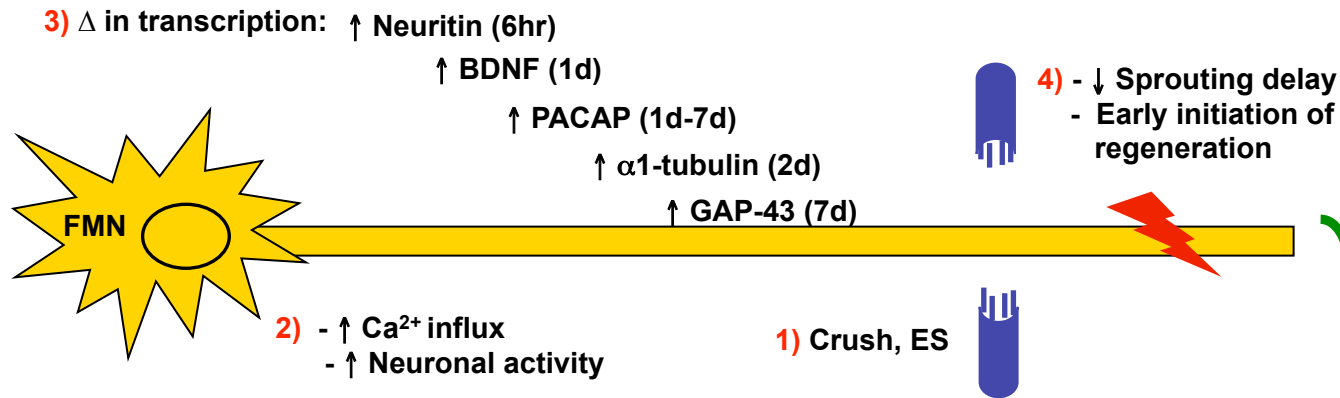
### **SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS**

#### **A. SUMMARY**

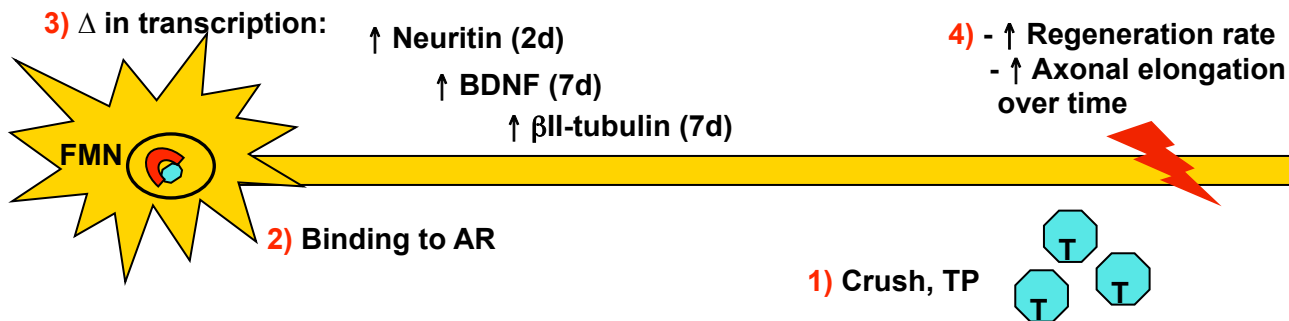
The overall findings of the present study are depicted in Figure 28. Aims 1 & 2 investigated the effects of ES and TP in the extratemporal crush model of facial nerve injury, and the results are summarized as follows:

- 1) Daily ES and systemic TP differentially enhanced facial nerve regenerative properties. While ES shortened the initial delay before sprout formation begins, TP accelerated the rate of axonal regeneration. Administration of the combination treatment of both had additive effects on regeneration, as the delay in sprouting was shortened and the rate was enhanced.
- 2) Daily ES and systemic TP differentially enhance expression of regeneration-associated genes. ES and TP targeted different genes, with ES upregulating expression of  $\alpha$ 1-tubulin and GAP-43 and TP upregulating expression of  $\beta$ -II tubulin. While both treatments upregulated expression of BDNF and neuritin, the effects of ES were rapid but transient and the effects of TP were delayed yet sustained. The combination of ES and TP induced both early and late upregulation as well as additive effects on expression of some genes such as PACAP.

## A. Electrical Stimulation



## B. Testosterone



**Figure 28. Working model of the effects of ES and TP treatments on axonal regeneration.** Effects of ES (A) and TP (B) are summarized in chronological steps from 1-4. Step #2 for both treatments has been demonstrated in previously published studies. FMN: facial motoneuron; crush; electrode; TP; AR

Aims 3 & 4 focused on development of a more clinically relevant, intratemporal crush model of facial nerve injury and investigation of the effects of prednisone, ES, and TP herein, as summarized below:

- 1) Compared to an extratemporal facial nerve crush, an intratemporal facial nerve crush in rats led to significantly delayed return of normal facial nerve function. Functional recovery and motor nerve conduction failed to return to normal at the end of the study period of 8 wpo. In addition, an intratemporal crush injury was associated with a ~85% cell survival, whereas an extratemporal crush injury produced a ~100% cell survival.
- 2) Administration of prednisone, ES, and TP had varying effects on functional recovery following an intratemporal crush. ES and TP improved recovery of facial functional parameters more than prednisone. Neither treatment by itself was able to shorten the time till complete functional recovery, as compared to no treatment. Only the combined administration of ES and TP was effective in accelerating complete recovery and returning motor nerve conduction to normal.

## B. CONCLUSIONS

The overall results of the present study, as described above, have been presented and discussed in the corresponding chapters. The following topics now incorporate the results together in context of current peripheral nerve regeneration research.

## 1. INSIGHT INTO MECHANISM OF ES-MEDIATED THERAPEUTIC EFFECTS

Previous studies have assessed whether ES enhances regeneration through a conditioning mechanism. A conditioning lesion, which precedes test injury, has been shown to accelerate rate of regeneration in concert with enhanced slow transport of cytoskeletal proteins (Bisby and Tetzlaff, 1992; McQuarrie 1986). Administration of ES 1 week prior to crush injury has no effect on promoting regeneration, suggesting that ES in the absence of injury does not alter the future behavior of motoneurons (Brushart et al., 2002). Also, unlike a conditioning lesion, ES promotes the onset of regeneration without increasing its speed. ES does, however, recruit more motoneurons to regenerate across the site of injury and enter the distal nerve stump (Brushart et al., 2002). Similarly, our results demonstrate that ES fails to accelerate the axonal elongation rate, but it significantly reduces the estimated delay before sprouting. Therefore, ES may serve as an effective treatment to initiate regeneration, followed by other treatments that speed and prolong axonal regeneration.

ES has previously been demonstrated to mediate its positive effects by directly affecting the cell body (Al-Majed et al., 2000a). Our results indicate that ES rapidly enhances expression of various regeneration-associated genes and thus may in turn enhance the motoneurons response to injury. The genes that were found to be upregulated play an important role in growth cone dynamics, axonal elongation, neuronal survival, neurite outgrowth, and target reinnervation. Activation of neurotrophin signaling is most likely an important result of ES treatment and precedes upregulation of cytoskeletal proteins (Al-Majed et al., 2000b; Al-Majed et al., 2004). Another important source of neurotrophic factors are denervated Schwann cells and the distal nerve stumps;

however, the upregulation of BDNF and GDNF at these sites has been reported to be delayed relative to the time of axotomy (Hoke et al., 2006; Boyd and Gordon, 2003a; Boyd and Gordon, 2003b). As this source of neurotrophic factors does not become available till later, the neuronal source is of critical importance during earlier times of axonal outgrowth. Hence, the immediate upregulation of neurotrophic molecules induced by ES is a likely mechanism of enhancing regeneration.

The ability of ES to initiate the onset of regeneration probably leads to earlier target muscle reinnervation. Gordon et al. (2007) have demonstrated that brief ES of the median nerve increases the number of motor units in the median nerve-innervated thenar muscles. Ahlborn et al. (2007) report that although ES considerably accelerates the rate of functional recovery following femoral nerve injury in mice, the overall functional outcome at the end of the study period is not considerable different between stimulated and unstimulated animals. Similarly, we also find that ES initiates return of facial function noticeably early but does not shorten the overall recovery period following intratemporal facial nerve injury.

## 2. INSIGHT INTO MECHANISM OF TP-MEDIATED THERAPEUTIC EFFECTS

A postulated mechanism by which gonadal steroids accelerate regeneration rate is by “priming” the cell body metabolically. Administering gonadal steroids to axotomized motoneurons at the time of injury may accelerate the genomic switch from a normal to a reparative mode, thereby enhancing the neuronal response to injury. A conditioning lesion similarly “primes” the neuron to enter a growth state prior to a testing lesion and results in an accelerated rate of peripheral nerve regeneration. The positive effects of a

conditioning lesion require an interval of days to weeks prior to the testing lesion.

For gonadal steroids, on the other hand, immediate exposure is required to accelerate regeneration, with delayed exposure having no effect (Kujawa and Jones, 1990). Therefore, gonadal steroids are likely to alter the synthetic capabilities of neurons more rapidly than a conditioning lesion.

Previous studies have demonstrated that the androgen-induced augmentation of facial nerve regeneration is AR-dependent. Administration of flutamide, a potent AR blocker that prevents binding of TP, prevents the increase in regeneration rate (Kujawa et al., 1995). Interestingly, axonal injury results in significant downregulation of AR in the FMN and SNB models (Drengler et al., 1997; Lubischer and Arnold, 1995). A potential explanation for this result is that injury induces transcription of cytoskeletal and growth-associated genes, while shutting down production of other genes such as AR that may be less necessary at the time. Therefore, the upregulation of regeneration-associated genes by TP may be mediated by preexisting steroid receptors. While alterations in genomic regulation are the classic mechanism of steroid action, recent studies have recently demonstrated that rapid, non-genomic mechanisms involving ion movements and/or initiation of signal transduction cascades may also underlie the observed effects on regeneration.

As testosterone can be aromatized to estrogen via the enzyme aromatase, the neurotherapeutic effects of TP observed in the present study may in part be mediated by its conversion to estradiol. Within the mammalian brain under normal conditions, aromatase is expressed mainly in neurons and at low levels in astrocytes (Balthazart and Ball, 1998; Negri-Cesi et al., 1992). Conditions of injury have been demonstrated to



increase the levels of aromatase in both neurons and glia (Zwain and Yen, 1999; Peterson et al., 2001). The dendritic morphology of rodent SNB has been shown to be disrupted by blocking aromatase (Burke et al., 1999). Recent studies from our lab demonstrate that following an extratemporal facial nerve injury in rats, administering DHT or estradiol (metabolites of testosterone) does not enhance functional recovery and regeneration rates to the same extent as TP (Foecking et al., 2009). As DHT is a non-aromatizable form of androgen, results suggest that a synergistic action of androgens and estrogens may be required to obtain maximal effects on regeneration events.

### 3. BENEFITS OF A COMBINATORIAL TREATMENT APPROACH

The present investigated a novel combinatorial treatment strategy based on the hypothesis that if the two treatments, ES and TP, work through different mechanisms, then together they might be used to obtain additive effects on regeneration. Our findings demonstrate that while ES enhances regeneration by reducing the delay in sprouting and rapidly upregulating gene expression, TP enhances regeneration by accelerating the axonal regeneration rate and prolonging expression of regeneration-associated genes. Therapeutically, a combination treatment may be effective because ES could be used to start up regeneration events, followed by TP to maintain the reparative process.

The need for a combinatorial treatment is especially evident as neither ES nor TP alone are effective in improving complete functional recovery in rats following a more proximal, intratemporal injury of the facial nerve. The finding that the combinatorial treatment of ES plus TP substantially shortens the recovery period may be due to ES recruiting more motoneurons to cross the crush zone early and TP maintaining expression

of neurotrophic factors and growth-associated genes long-term to promote target reinnervation. In addition, TP may also prevent muscle atrophy associated with prolonged target denervation. As ES has previously been reported to improve specificity of reinnervation, brief ES treatment may also enhance the outcome of an intratemporal facial nerve injury by reducing aberrant regeneration along the multiply branched motor nerve. Therefore, a combinatorial treatment strategy would be especially beneficial in cases of prolonged recovery periods following nerve injury.

#### 4. CLINICAL IMPLICATIONS OF THE INTRATEMPORAL FACIAL NERVE INJURY MODEL

Prior to this study, an animal model representing intratemporal facial nerve injury had not been developed. As intratemporal injuries of the facial nerve are more prevalent than injuries occurring after the nerve's exit from the skull, the model developed in the present study is of clinical importance. Furthermore, an intratemporal facial nerve crush, ~6-7 mm proximal to the extratemporal crush, leads to dramatically delayed facial functional recovery and return of normal motor nerve conduction. In a series of studies, Fu and Gordon (1995) provided evidence that the regenerative capacity of injured motoneurons regressively deteriorates with increased distance and time (Fu and Gordon 1995a; Fu and Gordon 1995b). Therefore, comparison of the extratemporal and intratemporal facial nerve injury models in the present study also supports the idea that there may be a limited window of opportunity for optimal nerve regeneration, which may be narrowed by an injury more proximal to the cell body.

Based on current findings, patients presenting with intratemporal vs. an extratemporal facial nerve injury can also be predicted to have delayed recovery from facial paralysis or increased chances of complications such as synkinesis. Although most studies have found that significant neuronal loss does not occur following crush injuries of the peripheral nerves, more proximal injuries and/or prolonged periods of target denervation may lead to some degree of cell loss as reported in our intratemporal facial nerve injury model. The ~15% cell loss observed may not account for the substantially delayed functional recovery. However, as full vibrissae movement is the main functional parameter whose recovery remains incomplete, the majority of the cell loss may be localized to FMN subnuclei projecting to the vibrissae muscles and thus may explain the lack of recovery. Investigating the amount of cell loss in different FMN subnuclei would shed additional insight into predicting the pathological result of proximal facial nerve injuries seen clinically. Also, the present studies assessed facial motoneuron survival at a maximum of 8 wpo; however, cell loss may further increase at later time points. If neuronal loss does play a critical role in preventing successful facial nerve regeneration, then therapies promoting long-term cell survival should be used in the clinical setting.

Lastly, the finding that prednisone appears to improve recovery of some but not all facial functional parameters undervalues its therapeutic use in crush or transection injuries of facial nerve. However, because of its anti-inflammatory actions, prednisone may nevertheless be important in cases of considerable facial nerve edema or inflammation. To promote successful regeneration after such injuries, prednisone would have to be combined with other treatments.

## 5. THERAPEUTIC NEED AND POTENTIAL FOR TRANSLATION OF THE ES PLUS TP TREATMENT STRATEGY

In comparison to animal models of peripheral nerve regeneration, regeneration in humans is known to be 3 times slower (Gordon et al., 2008). Given the ~3 mm/d regeneration rate for the fastest growing axons in rats, the expected regeneration rate in humans would be ~1 mm/d. Furthermore, the latency period for the axons to cross the site of injury and enter distal nerve stumps is also longer in humans. Not only is the inherent regeneration rate slower, but peripheral nerves in humans also have to grow over longer distances. Therefore, suboptimal functional recovery is often seen following peripheral nerve injuries, such as that of the facial nerve. The past quarter of a century has focused on optimization of surgical repair of injured nerves. However, therapies targeting molecular mechanisms of regeneration are lacking.

As ES and TP differentially enhance the intrinsic ability of neurons to regenerate, together they have the potential to be an effective treatment strategy. Several studies demonstrate translation of ES and TP treatments into clinic. Brief ES of the median nerve in patients with carpal tunnel syndrome has been shown to improve functional recovery (Gordon et al., 2007). Direct ES of the sacral and hypoglossal have also been conducted in patient trials for treatment of bladder dysfunction and obstructive sleep apnea, respectively (Sutherland et al., 2007; Schwartz et al., 2001). Therefore, ES for a brief duration represents a feasible approach for treatment of peripheral nerve injuries. Similarly, testosterone treatment has been tested in multiple patient trials. For example, an intramuscular testosterone treatment in men diagnosed with Parkinson's disease increased total testosterone levels by ~270% and improved non-motor symptoms of the

disease (Okun et al., 2006; Okun et al., 2002). Clinical trials in male multiple sclerosis patients have demonstrated that application of testosterone gel to the upper arms increased circulating hormone levels by an average of 50% and improved cognitive function, slowed brain atrophy rate, and induced production of neurotrophic factors (Sicotte et al., 2002; Gold et al., 2008). Sublingual administration of testosterone in healthy females also attenuates the integrated central stress response (Hermans et al., 2007).

Testosterone treatment in patients with peripheral nerve injuries has not yet been investigated. As different doses and routes of delivery for testosterone treatment are currently being used in patient trials, the most effective dosage and administration method would have to be determined. Our studies have demonstrated that endogenous testosterone levels in male hamsters are not sufficient to promote regeneration and that supraphysiological levels ( $>3$  times normal levels) are effective. Further studies in our animal models can determine the minimal TP dosage required to obtain enhanced effects on regenerative properties and functional recovery. Previous studies from our lab have demonstrated that following an extratemporal facial nerve crush in male hamsters, TP needs to be administered soon after injury in order to have beneficial effects (Tanzer and Jones, 2004). A 6 h delay in treatment prevents TP from augmenting regeneration. Furthermore, a 6 h immediate TP exposure enhanced regeneration and functional recovery to the same extent as a 7 d treatment. These findings have important clinical implications and suggest that an immediate but short-term treatment with gonadal steroids may have significant therapeutic advantages for peripheral nerve injuries.

### C. FUTURE DIRECTIONS

The present study offers insight into the effects of ES and TP on functional recovery and molecular mechanisms of regeneration in two facial nerve injury models. However, there are several questions that need to be answered and thus are presented as future directions below. These additional studies will further advance the translation of the proposed combinatorial treatment strategy (ES plus TP) into the clinic.

1. What is the minimal dose and duration of TP treatment required to achieve therapeutic effects?
2. Why is return of functional recovery and motor nerve conduction substantially delayed following an intratemporal vs. an extratemporal facial nerve crush injury?
  - a. Using retrograde dye labeling, determine the number of motoneurons that cross into the nerve segment distal to the crush site following an intratemporal vs. extratemporal injury.
  - b. Using retrograde dye labeling, determine the number of motoneurons that reinnervate vibrissal muscles following an intratemporal vs. extratemporal injury.
  - c. Determine facial motoneurons survival in different subnuclei of the FMN to assess if cell loss is greater in certain subpopulations.
3. Can the combinatorial treatment of ES plus TP attenuate the 15% cell loss induced by the intratemporal crush?

4. Do ES and TP enhance regeneration by altering subcellular trafficking between the cell body and the distal processes?
  - a. Determine whether ES and TP increase levels of axoplasmic proteins, such as importins, that are retrogradely transported after injury to modulate the regenerative response.
  - b. Determine the timing and degree of increase in levels of neurotrophic factors and growth-associated proteins in the nerve relative to the cell body following treatment with ES and TP.

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## VITA

The author, Nijee Sharma, was born to Sudhir and Anita Sharma in Nigeria on July 29, 1982. After spending most of her childhood in New Delhi, India, Nijee moved to Chicago, IL in 1994. She completed her secondary education at Maine East High School (Park Ridge, IL) and entered Lake Forest College (Lake Forest, IL) in 2000. While at Lake Forest College, Nijee served as a writing tutor and teaching assistant for a number of biology courses. She also undertook an internship at the Autism and Epilepsy Specialty Services of Illinois, where she worked as a clinical research assistant.

In 2002, Nijee joined the molecular neuroscience laboratory of Dr. Shubhik DebBurman, where she investigated protein misfolding linked to Parkinson's disease. She presented her research at various regional and national conferences and received awards for her poster presentations at the American Society for Biochemistry and Molecular Biology meeting (2004) and the Chicago Chapter of the Society for Neuroscience meeting (2003). Nijee went on to complete an undergraduate thesis that was given the Phi Beta Kappa Senior Thesis Award at Lake Forest College. Graduating Magna Cum Laude and with an Excellence in Science Award, Nijee entered the MD/PhD program at Loyola University Stritch School of Medicine in 2004. For her PhD track, she decided to join the Neuroscience Graduate Program.

During the summers of 2005 and 2006, Nijee worked with Dr. Nancy Muma to evaluate the role of estrogen receptors in serotonin related disorders. Later in 2006, she joined the laboratory of Dr. Kathryn Jones and became involved in peripheral nerve regeneration research, in collaboration with the Department of Otolaryngology – Head and Neck Surgery at Loyola University Medical Center. Nijee has presented her dissertation research at the local (Chicago Chapter) and national Society for Neuroscience meetings (2008). She also received a travel award from the American Association of Anatomists to present at the Experimental Biology meeting (2008). She is currently a student member of the American Association of Anatomists and the Society for Neuroscience. After graduating from the MD/PhD program in 2011, Nijee plans on pursuing a residency in neurology and becoming a liaison between medicine and scientific research.

#### Publications:

**Sharma N**, Moeller CM, Cunningham K, Marzo SJ, Jones KJ, and Foecking EM. (2010). Combinatorial treatments enhance recovery following facial nerve crush. Laryngoscope, 120, 1523-1530.

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Brandis K, Holmes I, England S, **Sharma N**, Kukreja L, and DebBurman SK. (2006). Alpha-synuclein fission yeast model: Concentration-dependent aggregation without membrane localization or toxicity. J. Mol. Neurosci., 28, 179-192.